

(19)



(11)

EP 3 248 984 A2

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:

29.11.2017 Bulletin 2017/48

(51) Int Cl.:

C07K 1/18 (2006.01)

(21) Application number: **17165390.0**

(22) Date of filing: **07.04.2017**

(84) Designated Contracting States:

**AL AT BE BG CH CY CZ DE DK EE ES FI FR GB
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO
PL PT RO RS SE SI SK SM TR**

Designated Extension States:

BA ME

Designated Validation States:

MA MD

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(30) Priority: **07.04.2016 JP 2016077127**

(54) **METHOD FOR PURIFYING PROTEIN, FUSION PROTEIN CONTAINING PEPTIDE TAG, AND PRODUCTION METHOD THEREOF**

(57) Disclosed is a method for purifying a protein, comprising steps of: preparing a sample containing a fusion protein containing an amino acid sequence of a peptide tag and an amino acid sequence of a target protein;

and separating contaminant proteins contained with the fusion protein in the sample and the fusion protein in the sample, wherein the peptide tag contains 12 or more acidic amino acid residues.

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Description

TECHNICAL FIELD

- 5 **[0001]** The present invention relates to a method for purifying a protein, a fusion protein containing a peptide tag, and a production method thereof.

BACKGROUND

- 10 **[0002]** According to the progress of molecular biology, a system for mass expressing an objective protein by introducing a recombinant gene encoding the objective protein into a host such as E. coli, a yeast or a cell has been developed. In such system, a sample prepared from the host contains contaminant proteins such as host-derived endogenous proteins, together with the objective protein. Thus, the objective protein needs to be further purified. As a method for purifying an objective protein, for example, a method for purifying VP1 protein by introducing eight glutamic acids and cysteine into
15 polyoma VP1 protein is disclosed in Stubenrauch K, et al, Purification of a viral coat protein by an engineered polyionic sequence. J. Chromatogr. B. Biomed. Sci. Appl: 737: 77-84, 2000.

SUMMARY OF THE INVENTION

- 20 **[0003]** An object of the present invention is to provide a purification method capable of easily obtaining high purity recombinant protein.
- [0004]** One aspect of the present invention is a method for purifying a protein, comprising steps of: preparing a sample comprising a fusion protein and contaminant proteins, the fusion protein comprising an amino acid sequence of a peptide tag and an amino acid sequence of a target protein, and separating the contaminant proteins and the fusion protein,
25 wherein the peptide tag comprises 12 or more acidic amino acid residues.
- [0005]** In particular embodiments, the peptide tag comprises any of the amino acid sequences of SEQ ID NOs. 1 to 8.
- [0006]** In particular embodiments, the peptide tag comprises 18 or more acidic amino acid residues.
- [0007]** In particular embodiments, the isoelectric point of the fusion protein is less than 6.
- [0008]** In particular embodiments, the salt concentration of the sample is 50 mM or more and 500 mM or less.
- 30 **[0009]** In particular embodiments, in the sample preparation step, a vector containing a polynucleotide encoding the fusion protein is introduced into a host cell, and the protein is expressed in the host cell to prepare the sample.
- [0010]** In particular embodiments, the fusion protein further comprises a cleavable site recognized by a protease, between the amino acid sequence of a peptide tag and the amino acid sequence of the target protein.
- [0011]** In particular embodiments, in the step of separating the contaminant proteins, the fusion protein and the con-
35 taminant proteins are separated using an ion exchange resin.
- [0012]** In particular embodiments, the ion exchange resin is an anion exchange resin.
- [0013]** In particular embodiments, the step of separating the contaminant proteins comprises passing the sample through an anion exchange resin to obtain (i) the anion exchange resin to which the fusion protein is bound and (ii) a flow-through fraction comprising the contaminant proteins, and eluting the fusion protein from the anion exchange resin
40 using a buffer with a salt concentration of 600 mM or more.
- [0014]** In particular embodiments, after the step of separating the contaminant proteins, the method further comprises a step of separating the fusion protein into the target protein and the peptide tag in a solution, using the protease recognizing the cleavable site.
- [0015]** In particular embodiments, in the step of separating the fusion protein, the solution comprising the separated
45 target protein and the separated peptide tag is passed through an anion exchange resin, and (i) the anion exchange resin to which the separated peptide tag is bound, and (ii) a flow-through fraction comprising the target protein, are obtained.
- [0016]** In particular embodiments, the salt concentration of the solution comprising the target protein and the peptide tag is 500 mM or less.
- 50 **[0017]** Another aspect of the present invention is a fusion protein comprising an amino acid sequence of a peptide tag and an amino acid sequence of a target protein, the peptide tag comprising 12 or more acidic amino acid residues.
- [0018]** In particular embodiment, the peptide tag comprises any of the amino acid sequences of SEQ ID NOs. 1 to 8.
- [0019]** In particular embodiments, the peptide tag comprises 18 or more acidic amino acid residues.
- [0020]** In particular embodiments, the isoelectric point of the fusion protein is less than 6.
- 55 **[0021]** In particular embodiments, the fusion protein further comprises a cleavable site recognized by a protease, between the amino acid sequence of the peptide tag and the amino acid sequence of the target protein.
- [0022]** Another aspect of the present invention is a method for producing the fusion protein, comprising steps of introducing a polynucleotide encoding the amino acid sequence of the peptide tag and a polynucleotide encoding the

amino acid sequence of the target protein into a host cell, and expressing the fusion protein in the host cell.

[0023] Another aspect of the present invention is a method for producing a pure target protein, the method comprising producing the fusion protein as described above, purifying the fusion protein by separating the contaminant proteins and the fusion protein and, after said purification, cleaving said fusion protein to obtain said target protein and separating said target protein from said peptide tag and optionally said enzyme used for said cleaving.

[0024] Another aspect of the present invention is use of a peptide tag comprising 12 or more acidic amino acid residues, for the above-mentioned method for purifying a protein.

[0025] A higher purity recombinant protein can be obtained by a simple method.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026]

Figs. 1A to 1C are diagrams schematically showing a principle of the purification methods provided herein, in which Fig. 1A shows a separation step, and Figs. 1B and 1C show a target protein acquisition step.

Fig. 2 is a figure showing the results of SDS (Sodium dodecyl sulfate)-polyacrylamide gel electrophoresis (SDS-PAGE) in Example 1.

Fig. 3 is a figure showing the results of SDS-PAGE in Example 2.

Fig. 4 is a figure showing the results of SDS-PAGE in Example 3.

Fig. 5 is a figure showing the results of SDS-PAGE in Example 4.

Fig. 6 is a figure showing the results of SDS-PAGE in Example 5.

Fig. 7 is a figure showing the results of SDS-PAGE in Example 6.

Fig. 8 is a figure showing the results of SDS-PAGE in Example 7.

Fig. 9 is a figure showing the results of SDS-PAGE in Example 8.

Fig. 10 is a figure showing the results of SDS-PAGE in Example 9.

Fig. 11 is a figure showing the results of SDS-PAGE in Example 10.

Fig. 12 is a figure showing the results of SDS-PAGE in Example 11.

Fig. 13 is a figure showing the results of SDS-PAGE in Example 12.

Fig. 14 is a figure showing the results of SDS-PAGE in Example 13.

Fig. 15 is a figure showing the results of SDS-PAGE in Comparative Example 1.

Fig. 16 is a figure showing the results of SDS-PAGE in Example 14.

Fig. 17 is a figure showing the results of SDS-PAGE in Example 15.

Fig. 18 is a figure showing the results of SDS-PAGE in Example 16.

Fig. 19 is a figure showing the results of SDS-PAGE in Example 17.

Fig. 20 is a figure showing the results of SDS-PAGE in Example 18.

Fig. 21 is a figure showing the results of SDS-PAGE in Example 19.

Fig. 22 is a figure showing the results of SDS-PAGE in Example 20.

Fig. 23 is a figure showing the results of SDS-PAGE in Example 21.

Fig. 24 is a figure showing the results of SDS-PAGE in Example 22.

Fig. 25 is a figure showing the results of SDS-PAGE in Example 23.

Fig. 26 is a figure showing the results of SDS-PAGE in Example 24.

Fig. 27 is a figure showing the results of SDS-PAGE in Example 25.

Fig. 28 is a figure showing the results of SDS-PAGE in Example 26.

Fig. 29 is a figure showing the results of SDS-PAGE in Example 27.

Fig. 30 is a figure showing the results of SDS-PAGE in Example 28.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0027] The purification methods of the present invention include steps of preparing a sample containing a fusion protein containing an amino acid sequence of a peptide tag and an amino acid sequence of a target protein and contaminant proteins (sample preparation step), and separating the fusion protein and the contaminant proteins (separation step). The contaminant protein means a protein such as host-derived endogenous proteins, other than the fusion protein and the target protein.

(Sample Preparation Step)

[0028] The fusion protein contains amino acid sequences of a peptide tag and a target protein. The peptide tag may be one containing 12 or more acidic amino acid residues. The number of the acidic amino acid residues in the peptide

tag is preferably 18 residues or more, more preferably 24 residues or more, further preferably 30 residues or more, and particularly preferably 36 residues or more, since separation from the contaminant proteins is improved, and an effect on separation due to the isoelectric point of the target protein, pH of the buffer and the like can be more suppressed, in the separation step. The upper limit of the number of the acidic amino acid residues is not particularly limited. The upper limit of the number of the acidic amino acid residues is preferably 100 residues or less, and more preferably 70 residues or less, from the viewpoint of the effect on the three-dimensional structure of the fusion protein and the like. The acidic amino acid residues contained in the peptide tag may be either one of an aspartic acid residue or a glutamic acid residue or both of them. The peptide tag may contain a neutral amino acid residue and/or a basic amino acid residue, other than the acidic amino acid residue. The ratio of the number of the acidic amino acid residues to the number of the amino acid residues of the total peptide tags is preferably 20% or more and more preferably 60% or more, since separation from the contaminant proteins is improved. In the sequence of the total peptide tags, the amino acid sequence containing the acidic amino acid residues may be a sequence in which all acidic amino acid residues are continuous, or one or a plurality of neutral amino acid residues and/or basic amino acid sequences may be interposed between the acidic amino acid residues. In particular embodiments, the peptide comprises at least 4 consecutive acidic amino acid residues. More particularly, the peptide comprises at least 6 consecutive acidic amino acid residues. Preferably the peptide comprises at least 6 consecutive aspartic acid residues and/or at least 6 consecutive glutamic acid residues or at least 12 consecutive acidic amino acids which are preferably selected from aspartic acid and/or glutamic acid. The isoelectric point (pI) of the peptide tag is preferably 5 or less and more preferably 4 or less, since separation from the contaminant proteins is improved, and the effects on separation due to the isoelectric point of the target protein, pH of the buffer and the like can be more suppressed. The isoelectric point is a pH value when the positive and negative ion concentrations in the aqueous solution become equal to each other. The isoelectric point can be measured, for example, by isoelectric electrophoresis, or the like. Specific examples of the peptide tag include the following peptide tags 1 to 8 (D; aspartic acid residue, E; glutamic acid residue).

<Peptide Tag>

[0029]

Peptide tag 1 EEEEEEDDDDDD (DE12; SEQ ID NO.: 1)

Peptide tag 2 EEEEEEEEDDDDDDDDD (DE18; SEQ ID NO.: 2)

Peptide tag 3 EEEEEEEEEEDDDDDDDDDDD (DE24; SEQ ID NO.: 3)

Peptide tag 4 EEEEEEEEEEEEDDDDDDDDDDDDD (DE30; SEQ ID NO.: 4)

Peptide tag 5 EEEEEEEEEEEEEEEEDDDDDDDDDDDDDDD (DE36; SEQ ID NO.: 5)

Peptide tag 6

NVEGKTGNATDEEEEEEEEEEEEEEDDDDDDDDDDDDEDSGAEIQDDDEE

GFDDEEEFDDDDDDDEHDDDDLENEENELEEELEERVEARKK (DED; SEQ ID NO.:

6)

Peptide tag 7 DLSNVEGKTGNATDEEEEEEEEEEEEEEDDDDDDDDDDDDEDSGAE (DES; SEQ ID NO.: 7)

Peptide tag 8 EEEEEEEEEEEEEEEEEEEEEEE (EO24; SEQ ID NO.: 8)

[0030] The target protein is not particularly limited. Examples of the target protein include proteins such as enzymes, receptors, interferons, interleukins, antibodies, and fluorescent proteins. The isoelectric point of the target protein is not particularly limited. Generally, the isoelectric point of a protein is 6 to 7, and even when any protein is targeted, the isoelectric point can be lowered by fusing a peptide tag, to an extent that the peptide tag can be sufficiently separated from the contaminant proteins.

[0031] In the fusion protein, the peptide tag may be bound to either N-terminal side or C-terminal side of the target protein. The peptide tag may be adjacently bound to the target protein, or other amino acid sequence may be inserted between the target protein and the peptide tag. For example, when an amino acid sequence of the cleavable site to be recognized by a protease is inserted between the amino acid sequences of the fusion protein and the peptide tag, it becomes easier to acquire a target protein by cleaving the peptide tag from the fusion protein. The isoelectric point of the fusion protein is not particularly limited. The isoelectric point of the fusion protein is preferably less than 6 and more preferably less than 5. The isoelectric point of many proteins is generally 6 to 7, thus when the isoelectric point of the

fusion protein is less than 6, separation of the fusion protein from the contaminant proteins becomes easier, and purity of the fusion protein can be improved.

[0032] The sample containing a fusion protein and contaminant proteins can be acquired by introducing a polynucleotide containing a base sequence encoding a peptide tag and a base sequence encoding a target protein into a host cell and expressing the fusion protein in the host cell. Expression of the fusion protein can be performed by a known genetic engineering method. A polynucleotide encoding a peptide tag and a polynucleotide encoding a target protein can be acquired by a known method such as chemical synthesis. The acquired polynucleotides can be amplified according to a known gene amplification method using these as templates. The amplified polynucleotide and an expression vector are treated with a restriction enzyme, and bound using an appropriate DNA ligase, whereby a recombinant expression vector containing the polynucleotides each encoding a peptide tag and a target protein can be constructed. In the construction of the expression vector, the base sequence encoding a peptide tag may be arranged at the 5' side or 3' side of the base sequence encoding a target protein. The base sequence encoding a peptide tag and the base sequence encoding a target protein may be adjacently arranged, or a base sequence encoding a cleavable site recognized by a protease or the like may be inserted there between. The host is not particularly limited. As the host, *E. coli*, a yeast, an insect cell, an insect body, an animal cell, a plant cell or the like can be used. The expression vector is not particularly limited. The expression vector includes plasmid vectors, phage vectors, virus vectors and the like. The expression vector can be properly selected depending on the used host. The expression vector may contain a regulatory sequence such as a replication origin, a promoter sequence or an enhancer sequence, or a sequence such as a selectable marker. Introduction of an expression vector into a host can be performed according to a known method depending on the host. Examples of the method include a calcium phosphate method, an electroporation method, a lipofection method, and the like. As described above, a transformant in which the expression vector is introduced into the host cell can be obtained. The obtained transformant is incubated under preferred conditions, thus a fusion protein can be produced.

[0033] After expressing the fusion protein, treatment such as extraction, crushing, centrifugation, solubilization and body fluid collection is performed on cells of the host, tissue, insect body, and the like, as necessary, to acquire a sample as a cell culture liquid, an extract, a homogenate, a solution, a body fluid, or the like. The sample contains contaminant proteins such as host-derived endogenous proteins, other than the produced fusion protein. For the preparation of the sample, a buffer such as a phosphate buffer, a citrate buffer, an acetate buffer, a tris buffer, a MOPS buffer or a HEPES buffer can be used. These buffers contain a salt with a weak acid or weak base used for obtaining a buffer action. In addition, salts such as sodium chloride, potassium chloride and calcium chloride can be used. The salt concentration of a buffer or sample herein means a concentration of a salt other than the salt intended for buffer action described above. The salt concentration of a sample is preferably 50 mM or more and 500 mM or less, and more preferably 50 mM or more and 250 mM or less, from the viewpoint of stability of the fusion protein, easiness of adjustment in the separation step, and the like. The pH of a sample is preferably 5 or more and 9 or less, and more preferably 6 or more and 8 or less, from the viewpoint of stability of the fusion protein, easiness of adjustment in the separation step, and the like.

[0034] When a sample is acquired using an *Escherichia coli* expression system, a plasmid vector can be used as an expression vector. Specific examples of the plasmid vector include pET, pGEX, pCold, pMAL, pCAL, and the like. The expression vector may contain a promoter sequence such as a replication origin, *lac*, T7 or *tac*, an enhancer sequence, or a sequence of a selectable marker such as ampicillin resistance gene or kanamycin resistance gene. Introduction of an expression vector into *E. coli* can be performed by a calcium phosphate method, an electroporation method, a lipofection method, and the like. After the introduction of an expression vector, *E. coli* is cultured in a medium containing the selectable marker, whereby a transformant in which the expression vector is introduced into *E. coli* can be selected. The thus-obtained transformant is proliferated under preferred conditions. Then, the proliferated transformant is expressed and induced depending on the selected promoter to produce a fusion protein. After expressing the fusion protein, a sample containing the fusion protein and contaminant proteins derived from *E. coli* can be acquired by crushing *E. coli* or the like.

[0035] When using an insect body such as silkworm or insect cells as an expression system, a transfer vector can be used. The transfer vector is transduced into the insect cells together with baculovirus DNA, and then an objective gene can be inserted into the baculovirus DNA by homologous recombination. Examples of the transfer vector include pM01, pM02, pHS01, pHS02, and the like. The transfer vector preferably contains a promoter such as a polyhedrin promoter or a p10 promoter. Examples of the insect cells include Sf9, Sf21, High5, TN-368, Bm5, and the like. Examples of the baculovirus include nuclear polyhedrosis virus (NPV), and specifically, AcMNPV, BmNPV and the like can be exemplified. The transfer vector and linearized baculovirus DNA are introduced into the insect cells, whereby homologous recombination occurs, and a recombinant baculovirus into which an objective gene is inserted is obtained. A virus solution containing a recombinant baculovirus is inoculated into an insect body or insect cells, or the like, whereby the recombinant baculovirus can be infected. The insect body such as silkworm may be in any form of imago, pupa, and larva. The insect body or insect cells are infected with the recombinant baculovirus, and bred or cultured for 1 to 7 days, whereby a fusion protein can be expressed. After expressing the fusion protein, a sample containing the fusion protein and contaminant proteins derived from the insect body or insect cells can be acquired by crushing the insect body or insect cells, or the like.

(Fusion Protein)

[0036] The fusion protein in the methods provided herein can be obtained, as the sample preparation step described above, by a production method including steps of introducing a polynucleotide containing a base sequence encoding an amino acid sequence of a peptide tag and a base sequence encoding an amino acid sequence of a fusion protein into a host cell, and expressing the fusion protein in the host cell. The fusion protein contains the amino acid sequence of a peptide tag containing 12 or more acidic amino acid residues and the amino acid sequence of a target protein. Examples of the fusion protein of the present invention include the following constructs 1 to 12 obtained by fusing NAD(P)H dehydrogenase, quinone 1 human (NQO1; pI = 8.91; SEQ ID NO.: 9) or Luciferase (pI = 6.71; SEQ ID NO.: 10) that is a target protein and the above peptide tags 1 to 8, and inserting a cleavable site recognized by protease HRV3C there between. The following construct 13 obtained by fusing HRV3C (pI = 8.46; SEQ ID NO.: 11) that is a target protein and the above peptide tag 7 is also exemplified.

Construct 1	DE12-HRV3Csite-NQO1 (SEQ ID NO.: 12)
Construct 2	DE18-HRV3Csite-NQO1 (SEQ ID NO.: 13)
Construct 3	DE24-HRV3Csite-NQO1 (SEQ ID NO.: 14)
Construct 4	DE30-HRV3Csite-NQO1 (SEQ ID NO.: 15)
Construct 5	DE36-HRV3Csite-NQO1 (SEQ ID NO.: 16)
Construct 6	DED-HRV3Csite-NQO1 (SEQ ID NO.: 17)
Construct 7	DES-HRV3Csite-NQO1 (SEQ ID NO.: 18)
Construct 8	EO24-HRV3Csite-NQO1 (SEQ ID NO.: 19)
Construct 9	NQO1-HRV3Csite-DED (SEQ ID NO.: 20)
Construct 10	NQO1-HRV3Csite-DES (SEQ ID NO.: 21)
Construct 11	DED-HRV3Csite-Luciferase (SEQ ID NO.: 22)
Construct 12	DES-HRV3Csite-Luciferase (SEQ ID NO.: 23)
Construct 13	DED-HRV3C (SEQ ID NO.: 24)

(Separation Step)

[0037] As to the sample containing the fusion protein obtained in the sample preparation step and the contaminant proteins, in the methods provided herein, the fusion protein and the contaminant proteins are separated. The phrase "the fusion protein and the contaminant proteins are separated" includes extracting and purifying the fusion protein, and storing into a container different from the contaminant proteins. The contaminant proteins may be substantially completely separated, or a part of the contaminant proteins may be separated. A part or all of the contaminant proteins is separated, whereby the purity of the fusion protein can be improved.

[0038] The separation means is not particularly limited, and a known separation means can be used. Separation by an ion exchange resin is preferred, and separation by an anion exchange resin is more preferred, since good separation from the contaminant proteins is obtained. The ion exchange group of the anion exchange resin is not particularly limited, and a quaternary ammonium (QA), a quaternary aminoethyl (QAE), diethylaminoethyl (DEAE) and the like can be used. Specific examples include Hitrap HP Q (GE Healthcare), TOYOPEARL GigaCap Q-650M, TOYOPEARL Q-600C AR, TOYOPEARL QAE-550, TOYOPEARL DEAE-650M, TOYOPEARL SuperQ-650M (all, Tosoh Corporation), Q101, DE101 (Mitsubishi Chemical Corporation), and the like. As a carrier of the ion exchange group, cellulose, dextran, agarose, a hydrophilic vinyl polymer or the like can be used. The sample containing the fusion protein obtained in the sample preparation step and the contaminant proteins is passed through such anion exchange resin to bind the fusion protein to the anion exchange resin. Thereafter, for example, the salt concentration of an eluate is increased to elute the contaminant proteins, then the fusion protein is eluted from the ion exchange resin with the eluate with a high salt concentration, whereby a purified fusion protein can be acquired. Examples of a method for increasing the salt concentration of an eluate include a stepwise method in which the salt concentration is stepwisely changed to elute an objective protein, a gradient method in which the salt concentration is continuously changed to elute an objective protein, and the like. As a buffer used for elution, a buffer such as a phosphate buffer, a citrate buffer, an acetate buffer, a tris buffer, a MOPS buffer or a HEPES buffer can be used. The salt concentration of the buffer is preferably set in the range of 50 mM or more and 2000 mM or less, and more preferably in the range of 50 mM or more and 1000 mM or less, from the viewpoint of separation from the contaminant proteins, stability of the fusion protein, and the like. Particularly, when the salt concentration of the buffer is set at 600 mM or more and more preferably 750 mM or more, separation from the contaminant proteins is improved. The range of pH of the buffer is not particularly limited. The range of pH of the buffer is preferably in the range of 5 or more and 9 or less, and more preferably in the range of 6 or more and 8 or less, from

the viewpoint of separation from the contaminant proteins, stability of the fusion protein, and the like.

[0039] Figs. 1A to 1C are schematic diagrams showing one embodiment, and Fig. 1A is a diagram showing a separation step. The principle of the separation step in the purification method of the present methods will be described based on this diagram. When a fusion protein is expressed in a host cell, a sample such as a disrupted cell suspension contains contaminant proteins 21A to 21F such as host-derived endogenous proteins, other than a fusion protein 20. The isoelectric points of the contaminant proteins 21 are high in the order of A to F. In a particular embodiment, a peptide tag 20b containing 12 or more acidic amino acid residues is bound to a target protein 20a to lower the isoelectric point of the fusion protein 20. The fusion protein 20 in which the isoelectric point is lowered can be held in an anion exchange resin 10 up to a high salt concentration. On the other hand, most of host-derived contaminant proteins cannot be held in the anion exchange resin 10 up to the same salt concentration. The fusion protein and the contaminant proteins are separated, based on the difference in the isoelectric points. Specifically, when a sample is brought into contact with the anion exchange resin 10, the contaminant protein 21A with a high isoelectric point passes without binding to the anion exchange resin. Other contaminant proteins and the fusion protein 20 bind to the anion exchange resin 10. When the salt concentration of the eluate is increased, contaminant proteins are eluted in the higher order of the isoelectric point (21B to 21D). The fusion protein 20 is eluted by an eluate with further higher salt concentration. This elution fraction contains contaminant proteins 21E and 21F with an isoelectric point close to that of the fusion protein 20. The salt concentration of the eluate is adjusted as described above, whereby many contaminant proteins can be separated, thus a high purity fusion protein can be obtained. The difference in the isoelectric points between the fusion protein and the contaminant proteins is relatively large, thus good separation is obtained also by a stepwise method in which the salt concentration is stepwisely changed to elute a protein.

(Target Protein Acquisition Step)

[0040] The fusion protein and the contaminant proteins contained in the sample can be separated as described above. When the fusion protein further contains a cleavable site to be recognized by a protease, the protease is reacted with the separated fusion protein, whereby a peptide tag is cleaved from the fusion protein, and only a target protein can be acquired. The purification methods provided herein, after the separation step, may further include a step of cleaving the peptide tag from the fusion protein in the solution, using a protease as described above, to acquire a target protein.

[0041] The kind of the protease is not particularly limited. Examples include HRV3C, PreScission protease, factor Xa, thrombin, TEV protease, and the like. For example, such protease is added to the eluate containing a target protein obtained in the separation step, whereby the peptide tag can be cleaved from the fusion protein. The reaction temperature and the reaction time can be properly set according to the kind of the protease and the like. In the solution after reaction, the target protein, the peptide tag and the protease are contained. The target protein can be separated from this solution, using a known separation means. The separation means is not particularly limited, and a known separation means such as various chromatography can be used. In particular embodiments, the separation step is based on the difference in the isoelectric points between the target protein and the peptide tag when the peptide tag is cleaved from the fusion protein. More particularly separation by an ion exchange resin using the difference in the isoelectric points is preferred, and separation by an anion exchange resin is further preferred. When a solution containing the target protein, the peptide tag and the protease after reaction by the protease is brought into contact with the anion exchange resin, the cleaved peptide tag binds to the ion exchange resin, and the target protein passes without binding to the ion exchange resin. Therefore, the target protein can be acquired by collecting this flow-through fraction. The salt concentration of the solution containing the target protein, the peptide tag and the protease is preferably 500 mM or less and more preferably 300 mM or less, since separation from the peptide tag is improved. The pH of the solution is preferably 5 or more and 9 or less, and more preferably 6 or more and 8 or less. As a buffer used for adjustments of the salt concentration and pH of the solution, a phosphate buffer, a citrate buffer, an acetate buffer, a tris buffer, a MOPS buffer, a HEPES buffer or the like can be used. Even when contaminant proteins remain in the solution fraction containing a fusion protein obtained in the separation step, the isoelectric point of those contaminant proteins is relatively close to that of the fusion protein. Therefore, the difference in the isoelectric point with the remaining contaminant proteins is also increased by cleaving the peptide tag from the fusion protein. Therefore, when using an ion exchange resin, separation from contaminant proteins is also improved, and a higher purity target protein can be obtained. The protease can be separated by a known separation means. When a difference is present in the isoelectric points of the target protein and the protease, both can be easily separated by an ion exchange resin.

[0042] Accordingly, in particular embodiments of the methods provided herein, the isoelectric point of the protease is preferably different from the isoelectric point of the target protein, preferably lower than that of the target protein. This makes it possible to easily separate the target protein and the protease, after cleaving the peptide tag with the protease. In particular embodiments this difference in isoelectric point is obtained by fusing the protease with a peptide tag. This makes it possible to adjust the isoelectric point of the protease to a desired value. As the peptide tag fused to the protease, the same one as the peptide tag to be bound to the fusion protein described above can be used. The amino acid sequence

of the peptide tag to be bound to the protease may be the same as or different from the amino acid sequence of the peptide tag to be bound to the fusion protein. The amino acid sequence of the peptide tag to be bound to the protease is preferably the same as the amino acid sequence of the peptide tag contained in the fusion protein. Thereby, the protease has the isoelectric point almost same as that of the fusion protein and also different from that of the target protein. The protease fused with the peptide tag preferably does not have a cleavable site to be recognized by this protease, in order to avoid decomposition by itself. When the protease fused with the peptide tag is used, the target protein, the cleaved peptide tag and the protease fused with the peptide tag are contained in the solution after reaction. This solution is passed through an anion exchange resin, whereby the protease fused with the peptide tag also binds to the anion exchange resin, together with the cleaved peptide tag, thus a step of separating the protease is not separately required.

[0043] The protease fused with the peptide tag can be obtained by using the protease as the target protein, in the expression of the fusion protein described above. The fusion protein and the protease can be expressed by introducing a vector into which a polynucleotide containing a base sequence encoding both amino acid sequences into a host. A vector into which a polynucleotide encoding the fusion protein is introduced and a vector into which a polynucleotide encoding the protease is inserted may be introduced into the host to express the fusion protein and the protease. The vector into which a polynucleotide encoding the fusion protein is inserted is introduced into the host, and the vector into which a polynucleotide encoding the protease is inserted is introduced into the host, and the fusion protein and the protease are expressed alone respectively, and then may be mixed. In this case, the hosts may be the same kind or the different kind, and are preferably the same kind, in the aspect of easiness of handling after expression and the like. The isoelectric point of the protease fused with the peptide tag is not particularly limited. The isoelectric point is preferably less than 6 and more preferably less than 5, since separation from the target protein is improved.

[0044] Based on the Figs. 1B and 1C, the principle of the target protein acquisition step in the purification methods provided herein will be described. The fusion protein 20 contains a cleavable site 20c to be recognized by the protease 22, between the target protein 20a and the peptide tag 20b. In a protease 22 fused with the peptide tag, a peptide tag 22b is bound to a protease 22a. When the fusion protein 20 and the protease 22 are reacted in the solution, the peptide tag 20b is cleaved from the fusion protein 20. The target protein 20a, the cleaved peptide tag 20b, the protease 22 fused with the peptide tag and contaminant proteins 21E and 21F remained in the separation step are contained in the solution after reaction. A peptide tag 20b with a low isoelectric point is cleaved, thus the isoelectric point of the target protein 20a becomes high as compared to that of the fusion protein 20. The contaminant proteins 21E and 21F have an isoelectric point relatively close to that of the fusion protein 20, thus the target protein 20a has an isoelectric point higher than those of those proteins. Furthermore, the target protein 20a has an isoelectric point higher than those of the cleaved peptide tag 20b and the protease 22 fused with the peptide tag 22b. Therefore, when this solution is diluted to lower the salt concentration, and passed through the anion exchange resin 10, the cleaved peptide tag 20b, the protease fused with the peptide tag 22 and contaminant proteins 21E and 21F bind to the anion exchange resin 10, but the target protein 20a passes through without binding thereto. High purity target protein 20a can be acquired by collecting this flow-through fraction.

EXAMPLES

[0045] Hereinbelow, the present invention will be further described in detail with reference to examples, but the present invention is not limited to these examples.

Example 1 [Expression and Purification of Fusion Protein DE12-HRV3Csite-NQO1 (Construct 1; pI = 5.84)]

[0046] Peptide tag 1 (DE12) was fused to the N-terminal side of NAD(P)H dehydrogenase, quinone 1 human (NQO1; pI = 8.91) that is a target protein, and expression and purification of a fusion protein containing a cleavable site HRV3Csite recognized by protease HRV3C between NQO1 and DE12 were performed.

(1) Construction of pM01_DE12 Vector

[0047] A pM01_DE12 vector in which the peptide tag DE12 was inserted into a pM01 vector was constructed.

[0048] Polynucleotides encoding DE12 (SEQ ID NOs.: 25 and 26) were annealed in pairs to prepare an insert part. A NheI site of the pM01 vector (SYSMEX CORPORATION) was treated with NheI (TAKARA BIO INC.), and the insert part was inserted using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.). Stellar Competent Cells were transformed with the constructed vector, then the obtained transformant was seeded on a LBA plate, and cultured at 37°C for 16 hrs. An ampicillin resistant transformant was selected from the obtained transformants according to normal procedure, and purification of plasmid was performed. In order to confirm a base sequence of the selected clone, the PCR reaction was performed by Big Dye Terminator v3.1 (Thermo Fisher Scientific Inc.), using primer 1 (SEQ ID NO.: 27) and primer 2

(SEQ ID NO.: 28) for sequence confirmation, then the base sequence was analyzed by a DNA sequencer (Thermo Fisher Scientific Inc.). A vector into which the insert part was introduced, that had no variation in other part, was defined as pM01_DE12.

5 (2) Construction of pM01_DE12_HRV3Csite_NQO1

[0049] An expression vector pM01_DE12_HRV3Csite_NQO1 into which a polynucleotide (SEQ ID NO.: 29) encoding NQO1 (SEQ ID NO.: 9) was inserted into pM01_DE12 was constructed.

[0050] The polynucleotide encoding NQO1 and primers 3F (SEQ ID NO.: 30) and 3R (SEQ ID NO.: 31) corresponding to the insert part were prepared, and the insert part was amplified using KOD-Plus- (TOYOBO CO., LTD.) as a PCR enzyme, under the following PCR conditions. The amplified PCR product was migrated by 1.0% (w/v) agarose electrophoresis, and the part matched to the length of the insert part was cut out from the gel and purified to prepare an insert part. A SmaI site of the pM01_DE12 vector was treated with SmaI (TAKARA BIO INC.), and the insert part was inserted using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.). Stellar Competent Cells (Clontech Laboratories, Inc.) were transformed with the constructed vector, then the obtained transformant was seeded on a LBA plate. The transformant was cultured at 37°C for 16 hrs. An ampicillin resistant transformant was selected from the obtained transformants according to normal procedure, and purification of plasmid was performed. In order to confirm a base sequence of the selected clone, the PCR reaction was performed by Big Dye Terminator v3.1 (Thermo Fisher Scientific Inc.), using primers 1 and 2 for sequence confirmation, then the base sequence was analyzed by a DNA sequencer (Thermo Fisher Scientific Inc.). A vector into which the insert part was introduced, that had no variation in other part, was defined as pM01_DE12_HRV3Csite_NQO1.

<PCR Conditions>

[0051] Reaction 1: 94.0°C, 2-minute reaction 2: 94.0°C, 15-second reaction 3: 52.0°C, 30-second reaction 4: 68.0°C, 1-minute reaction 5: reaction 2 to reaction 4 are repeated 30 times per 1 kbp

(3) Preparation of Recombinant Baculovirus and Expression of Fusion Protein DE12-HRV3Csite-NQO1

[0052] pM01_DE12_HRV3Csite_NQO1 and baculovirus DNA were introduced into silkworm culture cells (BmN cells), and homologous recombination of virus was performed. The culture cells were cultured for 6 days, and the recombinant virus was collected. Thereafter, a virus solution diluted 50 times with MilliQ water was inoculated into pupa of silkworm, and the pupa was incubated for 6 days.

35 (4) Purification of DE12-HRV3Csite-NQO1

[0053] After incubation, the pupa of silkworm was collected, and 50 mL of a buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 tablet Complete EDTA free (Roche), phenylthiourea) was added per a pupa to homogenate the pupa. The solution was centrifuged (8,000 g, 10 minutes), then the supernatant fraction was filtered with 0.8 µm of a filter agent, and the filtrate was collected. The collected filtrate was loaded on an ion exchange resin (Hitrap Q HP (1 mL)), and purified using a mixed solution of 20 mM Tris-HCl (pH 8.0, 150 mM NaCl; buffer A) and 20 mM Tris-HCl (pH 8.0, 1000 mM NaCl; buffer B). First, only buffer A (NaCl concentration of 150 mM) was flown through the ion exchange resin (EL0), and a fraction eluted with a 3:1 mixed solution of buffer A:buffer B (NaCl concentration of 363 mM) (EL1), a fraction eluted with a 1:1 mixed solution of buffer A:buffer B (NaCl concentration of 575 mM) (EL2), a fraction eluted with a 1:3 mixed solution of buffer A:buffer B (NaCl concentration of 788 mM) (EL3), and finally, a fraction eluted with only buffer B (NaCl concentration of 1000 mM) (EL4) were each collected.

(5) Detection of DE12-HRV3Csite-NQO1

[0054] SDS (Sodium dodecyl sulfate)-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 5 to 20% gradient gel and an electrophoresis apparatus (APRO Science Inc.). A sample and β-ME Sample Treatment for Tris SDS (COSMO BIO Co., Ltd. were mixed in a volume ratio of 1:1, and treated at 100°C for 3 minutes, then 5 µL of the resulting solution was loaded on the gel. As a molecular weight marker, 2.5 µL of Blue Star (NIPPON Genetics Co., Ltd.) was loaded on the gel. The gel loaded with the sample was migrated at a voltage of 400 V for 14 minutes, and then transferred to a membrane using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc.). The transferred membrane was set in i-Bind system (Thermo Fisher Scientific Inc.), and an antigen-antibody reaction was performed. In the antigen-antibody reaction, an ANTI-FLAG (registered trademark) M2-Peroxidase (HRP) antibody (Merck & Co., Inc.) or an Anti-NQO1 antibody (Cell Signaling Technology, Inc.) and Anti-IgG (H+L chain) (Mouse) pAb-HRP (Beckman Coulter,

Inc.) were diluted 2000 times and used. Also, Luminata Forte (Merck & Co., Inc.) as a HRP reagent for detection and Gel Doc XR+ system (Bio-Rad Laboratories, Inc.) as a detector were used.

[0055] After performing western blotting, the used membrane was stained by GelCode Blue Stain Reagent (Thermo Fisher Scientific Inc.), and excess staining was bleached, then the image was photographed using Gel Doc XR+ system (Bio-Rad Laboratories, Inc.). The results were shown in Fig. 2. In Fig. 2, the results of electrophoresis are shown as follows; M: molecular weight marker, lane 1: homogenate solution, lane 2: supernatant fraction after centrifugation, lane 3: precipitated fraction after centrifugation, lanes 4 to 7: flow-through fractions 1 to 4 of the ion exchange resin, lane 8: elution fraction by buffer A (NaCl concentration of 150 mM) (EL0), lane 9: elution fraction by a 3:1 mixed solution of buffer A:buffer B (NaCl concentration of 363 mM) (EL1), lane 10: elution fraction by a 1:1 mixed solution of buffer A:buffer B (NaCl concentration of 575 mM) (EL2), lane 11: elution fraction by a 1:3 mixed solution of buffer A:buffer B (NaCl concentration of 788 mM) (EL3), and lane 12: elution fraction by buffer B (NaCl concentration of 1000 mM) (EL4). The part surrounded by a broken line shows a band containing the target protein. Explanation of each lane is common in Figs. 3 to 15.

Example 2 [Expression and Purification of Fusion Protein DE18-HRV3Csite-NQO1 (Construct 2; pl = 5.03)]

[0056] Peptide tag 2 (DE18) was fused to the N-terminal side of NQO1 that is a target protein, and expression and purification of a fusion protein containing HRV3Csite between NQO1 and DE18 were performed.

[0057] The same procedures were carried out as in Example 1, except for preparing an insert part using polynucleotides (SEQ ID NOs.: 32 and 33) encoding DE18 (SEQ ID NO.: 2) in place of the polynucleotides encoding the peptide tag DE12, to construct a pM01_DE18 vector.

[0058] Further, pM01_DE18_HRV3Csite_NQO1 was constructed, and a recombinant baculovirus was prepared in the same manner as in Example 1, then expression, purification and detection of fusion protein DE18-HRV3Csite-NQO1 were performed. The results were shown in Fig. 3.

Example 3 [Expression and Purification of Fusion Protein DE24-HRV3Csite-NQO1 (Construct 3; pl = 4.78)]

[0059] Peptide tag 3 (DE24) was fused to the N-terminal side of NQO1 that is a target protein, and expression and purification of a fusion protein containing HRV3Csite between NQO1 and DE24 were performed.

(1) Construction of pM01_DE24 Vector

A pM01_DE24 vector in which a polynucleotide encoding peptide tag DE24 was inserted into a pM01 vector was constructed as below.

The polynucleotide (SEQ ID NO.: 34) encoding peptide tag 6 (DED; SEQ ID NO.: 6) and primers 4F (SEQ ID NO.: 35) and 4R (SEQ ID NO.: 36) corresponding to the insert part were prepared, and the insert part was amplified using KOD-Plus- (TOYOBO CO., LTD.) as a PCR enzyme, under the same PCR conditions as in Example 1. The amplified PCR product was migrated by 1.0% (w/v) agarose electrophoresis, and the part matched to the length of the insert part was cut out from the gel and purified to prepare an insert part. A SmaI site of the pM01 vector (SYSMEX CORPORATION) was treated with SmaI (TAKARA BIO INC.), and the insert part was inserted using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.). Stellar Competent Cells (Clontech Laboratories, Inc.) were transformed with the constructed vector, then the obtained transformant was seeded on a LBA plate. The transformant was cultured at 37°C for 16 hrs. An ampicillin resistant transformant was selected from the obtained transformants according to normal procedure, and purification of plasmid was performed. In order to confirm a base sequence of the selected clone, the PCR reaction was performed by Big Dye Terminator v3.1 (Thermo Fisher Scientific Inc.), using primers 1 and 2 for sequence confirmation, then the base sequence was analyzed by a DNA sequencer (Thermo Fisher Scientific Inc.). A vector into which the insert part was introduced, that had no variation in other part, was defined as pM01_DE24.

(2) pM01_DE24_HRV3Csite_NQO1 was constructed, and a recombinant baculovirus was prepared in the same manner as in Example 1, then expression, purification and detection of fusion protein DE24-HRV3Csite-NQO1 were performed. The results were shown in Fig. 4.

Example 4 [Expression and Purification of Fusion Protein DE30-HRV3Csite-NQO1 (Construct 4; pl = 4.61)]

[0060] Peptide tag 4 (DE30) was fused to the N-terminal side of NQO1 that is a target protein, and expression and purification of a fusion protein containing HRV3Csite between NQO1 and DE30 were performed.

[0061] The same procedures were carried out as in Example 1, except for preparing an insert part using polynucleotides (SEQ ID NOs.: 37 and 38) encoding DE30 (SEQ ID NO.: 4) in place of the polynucleotides encoding the peptide tag DE12, to construct a pM01_DE30 vector.

[0062] Further, pM01_DE30_HRV3Csite_NQO1 was constructed, and a recombinant baculovirus was prepared in the same manner as in Example 1, then expression, purification and detection of fusion protein DE30-HRV3Csite-NQO1 were performed. The results were shown in Fig. 5.

Example 5 [Expression and Purification of Fusion Protein DE36-HRV3Csite-NQO1 (Construct 5; pI = 4.49)]

[0063] Peptide tag 5 (DE36) was fused to the N-terminal side of NQO1 that is a target protein, and expression and purification of a fusion protein containing HRV3Csite between NQO1 and DE36 were performed.

[0064] The same procedures were carried out as in Example 1, except for preparing an insert part using polynucleotides (SEQ ID NOs.: 39 and 40) encoding DE36 (SEQ ID NO.: 5) in place of the polynucleotides encoding the peptide tag DE12, to construct a pM01_DE36 vector.

[0065] Further, pM01_DE36_HRV3Csite_NQO1 was constructed, and a recombinant baculovirus was prepared in the same manner as in Example 1, then expression, purification and detection of fusion protein DE36-HRV3Csite-NQO1 were performed. The results were shown in Fig. 6.

Example 6 [Expression and Purification of Fusion Protein DED-HRV3Csite-NQO1 (Construct 6; pI = 4.26)]

[0066] Peptide tag 6 (DED) was fused to the N-terminal side of NQO1 that is a target protein, and expression and purification of a fusion protein containing HRV3Csite between NQO1 and DED were performed.

(1) Construction of pHS01_DED Vector

A pHS01_DED vector in a peptide tag DED (SEQ ID NO.: 6) was inserted into a pHS01 vector was constructed as below.

The polynucleotide (SEQ ID NO.: 34) encoding the peptide tag DED and primers 5F (SEQ ID NO.: 41) and 5R (SEQ ID NO.: 42) corresponding to the insert part were prepared, and the insert part was amplified using KOD-Plus-(TOYOBO CO., LTD.) as a PCR enzyme, under the same PCR conditions as in Example 1. The amplified PCR product was migrated by 1.0% (w/v) agarose electrophoresis, and the part matched to the length of the insert part was cut out from the gel and purified to prepare an insert part. A NheI site of the pHS01 vector (SYSMEX CORPORATION) was treated with NheI (TAKARA BIO INC.), and the insert part was inserted using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.). Stellar Competent Cells were transformed with the constructed vector, then the obtained transformant was seeded on a LBA plate. The transformant was cultured at 37°C for 16 hrs. An ampicillin resistant transformant was selected from the obtained transformants according to normal procedure, and purification of plasmid was performed. In order to confirm a base sequence of the selected clone, the PCR reaction was performed by Big Dye Terminator v3.1 (Thermo Fisher Scientific Inc.), using primers 1 and 2 for sequence confirmation, then the base sequence was analyzed by a DNA sequencer (Thermo Fisher Scientific Inc.). A vector into which the insert part was introduced, that had no variation in other part, was defined as pHS01_DED.

(2) HS01_DED_HRV3Csite_NQO1 was constructed, and a recombinant baculovirus was prepared in the same manner as in Example 1, then expression, purification and detection of fusion protein DED-HRV3Csite-NQO1 were performed. The results were shown in Fig. 7.

Example 7 [Expression and Purification of Fusion Protein DES-HRV3Csite-NQO1 (Construct 7; pI = 4.55)]

[0067] Peptide tag 7 (DES) was fused to the N-terminal side of NQO1 that is a target protein, and expression and purification of a fusion protein containing HRV3Csite between NQO1 and DES were performed.

[0068] The same procedures were carried out as in Example 6, except for using primers 6F (SEQ ID NO.: 43) and 6R (SEQ ID NO.: 44) as the primers corresponding to the insert part, to construct pHS01_DES.

[0069] pHS01_DES_HRV3Csite_NQO1 was constructed, and a recombinant baculovirus was prepared in the same manner as in Example 1, then expression, purification and detection of fusion protein DES-HRV3Csite-NQO1 were performed. The results were shown in Fig. 8.

Example 8 [Expression and Purification of Fusion Protein EO24-HRV3Csite-NQO1 (Construct 8; pI = 4.73)]

[0070] Peptide tag 8 (EO24) was fused to the N-terminal side of NQO1 that is a target protein, and expression and purification of a fusion protein containing HRV3Csite between NQO1 and EO24 were performed.

(1) Construction of pHS01_EO24

Polynucleotides encoding peptide tag EO24 (SEQ ID NOs.: 45 and 46) were annealed in pairs to prepare an insert part. A NheI site of the pHS01 vector (SYSMEX CORPORATION) was treated with NheI (TAKARA BIO INC.), and

the insert part was inserted using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.). Stellar Competent Cells (Clontech Laboratories, Inc.) were transformed with the constructed vector, then the obtained transformant was seeded on a LBA plate. The transformant was cultured at 37°C for 16 hrs. An ampicillin resistant transformant was selected from the obtained transformants according to normal procedure, and purification of plasmid was performed.

In order to confirm a base sequence of the selected clone, the PCR reaction was performed by Big Dye Terminator v3.1 (Thermo Fisher Scientific Inc.), using primers 1 and 2 for sequence confirmation, then the base sequence was analyzed by a DNA sequencer (Thermo Fisher Scientific Inc.). A vector into which the insert part was introduced, that had no variation in other part, was defined as pHS01_EO24.

(2) pHS01_EO24_HRV3Csite_NQO1 was constructed, and a recombinant baculovirus was prepared in the same manner as in Example 1, then expression, purification and detection of fusion protein EO24-HRV3Csite-NQO1 were performed. The results were shown in Fig. 9.

Example 9 [Expression and Purification of Fusion Protein NQO1-HRV3Csite-DED (Construct 9; pl = 4.26)]

[0071] The peptide tag DED was fused to the C-terminal side of NQO1 that is a target protein, and expression and purification of a fusion protein containing HRV3Csite between NQO1 and DED were performed.

(1) Construction of pHS02_DED Vector

pHS02_DE12 Vector in which a polynucleotide encoding the peptide tag DED was inserted into pHS02 vector was constructed as below.

The polynucleotide (SEQ ID NO.: 34) encoding the peptide tag DED and primers 7F (SEQ ID NO.: 47) and 7R (SEQ ID NO.: 48) corresponding to the insert part were prepared, and the insert part was amplified using KOD-Plus- (TOYOBO CO., LTD.) as a PCR enzyme, under the same PCR conditions as in Example 1. The amplified PCR product was migrated by 1.0% (w/v) agarose electrophoresis, and the part matched to the length of the insert part was cut out from the gel and purified to prepare an insert part. A NheI site of the pHS02 vector (SYSMEX CORPORATION) was treated with NheI (TAKARA BIO INC.), and the insert part was inserted using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.). Stellar Competent Cells (Clontech Laboratories, Inc.) were transformed with the constructed vector, then the obtained transformant was seeded on a LBA plate. The transformant was cultured at 37°C for 16 hrs. An ampicillin resistant transformant was selected from the obtained transformants according to normal procedure, and purification of plasmid was performed. In order to confirm a base sequence of the selected clone, the PCR reaction was performed by Big Dye Terminator v3.1 (Thermo Fisher Scientific Inc.), using primers 1 and 2 for sequence confirmation, then the base sequence was analyzed by a DNA sequencer (Thermo Fisher Scientific Inc.). A vector into which the insert part was introduced, that had no variation in other part, was defined as pHS02_DED.

(2) The same procedures were carried out as in Example 1, except for using primers 8F (SEQ ID NO.: 49) and 8R (SEQ ID NO.: 50) as the primers corresponding to the insert part, to construct pHS02_NQO1_HRV3Csite_DED.

(3) Further, a recombinant baculovirus was prepared in the same manner as in Example 1, and expression, purification and detection of fusion protein NQO1-HRV3Csite-DED were performed. The results were shown in Fig. 10.

Example 10 [Expression and Purification of Fusion Protein NQO1-HRV3Csite-DES (Construct 10; pl = 4.55)]

[0072] Peptide tag DES was fused to the C-terminal side of NQO1 that is a target protein, and expression and purification of a fusion protein containing HRV3Csite between NQO1 and DES were performed.

(1) The same procedures were carried out as in Example 9, except for using 9F (SEQ ID NO.: 51) and 9R (SEQ ID NO.: 52) as the primers, to construct pHS02_DES.

(2) The same procedures were carried out as in Example 9 to construct pHS01_NQO1_HRV3Csite_DES.

(3) A recombinant baculovirus was prepared in the same manner as in Example 1, and expression, purification and detection of fusion protein NQO1-HRV3Csite-DES were performed. The results were shown in Fig. 11.

Example 11 [Expression and Purification of Fusion Protein DED-HRV3Csite-Luciferase (Construct 11; pl = 4.47)]

[0073]

(1) Construction of pHS01_DED Vector

The same procedures were carried out as in Example 6 to construct pHS01_DED.

(2) Construction of pHS01_DED_HRV3Csite_Luciferase

The polynucleotide (SEQ ID NO.: 53) encoding Luciferase and primers 10F (SEQ ID NO.: 54) and 10R (SEQ ID

NO.: 55) corresponding to the insert part were prepared, and the insert part was amplified using KOD-Plus- (TOYOBO CO., LTD.) as a PCR enzyme, under the same PCR conditions as in Example 1. The amplified PCR product was migrated by 1.0% (w/v) agarose electrophoresis, and the part matched to the length of the insert part was cut out from the gel and purified to prepare an insert part. A SmaI site of the pHS01_DED vector was treated with SmaI (TAKARA BIO INC.), and the insert part was inserted using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.). Stellar Competent Cells (Clontech Laboratories, Inc.) were transformed with the constructed vector, then the obtained transformant was seeded on a LBA plate. The transformant was cultured at 37°C for 16 hrs. An ampicillin resistant transformant was selected from the obtained transformants according to normal procedure, and purification of plasmid was performed. In order to confirm a base sequence of the selected clone, the PCR reaction was performed by Big Dye Terminator v3.1 (Thermo Fisher Scientific Inc.), using primers 1 and 2 for sequence confirmation, then the base sequence was analyzed by a DNA sequencer (Thermo Fisher Scientific Inc.). A vector into which the insert part was introduced, that had no variation in other part, was defined as pHS01_DED_HRV3Csite_Luciferase. (3) A recombinant baculovirus was prepared in the same manner as in Example 1, and expression, purification and detection of fusion protein DED-HRV3Csite-Luciferase were performed. The results were shown in Fig. 12.

Example 12 [Expression and Purification of Fusion Protein DES-HRV3Csite-Luciferase (Construct 12; pl = 4.75)]

[0074] The same procedures were carried out as in Example 11, except for using a pHS01_DES vector constructed in Example 7 in place of the pHS01_DED vector, to construct pHS02_DES_HRV3Csite_Luciferase.

[0075] A recombinant baculovirus was prepared in the same manner as in Example 1, and expression, purification and detection of fusion protein DES-HRV3Csite-Luciferase were performed. The results were shown in Fig. 13.

Example 13 [Expression and Purification of Fusion Protein DED-HRV3C (Construct 13; pl = 4.75)]

[0076]

(1) Construction of pET17b_DED_HRV3C

The polynucleotide (SEQ ID NO.: 34) encoding the peptide tag DED, a polynucleotide (SEQ ID NO.: 56) encoding HRV3C, primers 11F and 11R (SEQ ID NOs.: 57 and 58; DED) corresponding to the insert part and primers 12F and 12R (SEQ ID NOs.: 59 and 60; HRV3C) were prepared, and the insert part was amplified using KOD-Plus- (TOYOBO CO., LTD.) as a PCR enzyme, under the same PCR conditions as in Example 1. The amplified PCR product was migrated by 1.0% (w/v) agarose electrophoresis, and the part matched to the length of the insert part was cut out from the gel and purified to prepare an insert part. A SmaI site and a NheI site of the pHS01 vector (SYSMEX CORPORATION) were treated with SmaI (TAKARA BIO INC.) and NheI (TAKARA BIO INC.), and the two insert parts were inserted using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.). Stellar Competent Cells (Clontech Laboratories, Inc.) were transformed with the constructed vector, then the obtained transformant was seeded on a LBA plate. The transformant was cultured at 37°C for 16 hrs. An ampicillin resistant transformant was selected from the obtained transformants according to normal procedure, and purification of plasmid was performed. In order to confirm a base sequence of the selected clone, the PCR reaction was performed by Big Dye Terminator v3.1 (Thermo Fisher Scientific Inc.), using primers 1 and 2 for sequence confirmation, then the base sequence was analyzed by a DNA sequencer (Thermo Fisher Scientific Inc.). A vector into which the insert part was introduced, that had no variation in other part, was defined as pHS01_DED_HRV3C.

Next, an insert for pET17b was prepared. Primers 13F (SEQ ID NO.: 61) and 13R (SEQ ID NO.: 62) were prepared, and the primers were amplified using pHS01_DED_HRV3C as a template, and using KOD-Plus- (TOYOBO CO., LTD.) as a PCR enzyme, under the same PCR conditions as in Example 1. The amplified PCR product was migrated by 1.0% (w/v) agarose electrophoresis, and the part matched to the length of the insert part was cut out from the gel and purified to prepare an insert part. A NdeI site of the pET17b Vector (Novagen, Inc.) was treated with NdeI (TAKARA BIO INC.), and the insert part was inserted using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.). Stellar Competent Cells (Clontech Laboratories, Inc.) were transformed with the constructed vector, then the obtained transformant was seeded on a LBA plate. The transformant was cultured at 37°C for 16 hrs. An ampicillin resistant transformant was selected from the obtained transformants according to normal procedure, and purification of plasmid was performed. In order to confirm a base sequence of the selected clone, the PCR reaction was performed by Big Dye Terminator v3.1 (Thermo Fisher Scientific Inc.), using primers 14 (SEQ ID NO.: 63) and 15 (SEQ ID NO.: 64) for sequence confirmation, then the base sequence was analyzed by a DNA sequencer (Thermo Fisher Scientific Inc.). A vector into which the insert part was introduced, that had no variation in other part, was defined as pET17b_DED_HRV3C.

(2) Expression and Purification of DED-HRV3C

E. coli was transformed using pET17b_DED_HRV3C, and 1.5 L of an LB medium containing ampicillin was cultured

at 37°C and cooled to 15°C around a turbidity OD of about 0.6, then IPTG was added thereto to induce expression of protein. After 16 hours, bacteria were collected by centrifugation (8,000 g, 15 minutes) and frozen at -80°C. 120 mL of a buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 tablet Complete EDTA free (Roche)) was added to the frozen *E. coli*, and *E. coli* was crushed by ultrasonic wave. The crushed solution was centrifuged (15,000 g x 30 minutes), then the supernatant fraction was filtered with 0.8 µm of a filter agent, and the filtrate was collected. The filtrate was loaded on an ion exchange resin (Hitrap Q HP (1 mL)), and purified using a mixed solution of 20 mM Tris-HCl (pH 8.0, 150 mM NaCl; buffer A) and 20 mM Tris-HCl (pH 8.0, 1000 mM NaCl; buffer B). First, only buffer A (NaCl concentration of 150 mM) was flown through the ion exchange resin (EL0), and a fraction eluted with a 3 : 1 mixed solution of buffer A:buffer B (NaCl concentration of 363 mM) (EL1), a fraction eluted with a 1 : 1 mixed solution of buffer A : buffer B (NaCl concentration of 575 mM) (EL2), a fraction eluted with a 1 : 3 mixed solution of buffer A : buffer B (NaCl concentration of 788 mM) (EL3), and finally, a fraction eluted with only buffer B (NaCl concentration of 1000 mM) (EL4) were each collected.

(3) The same procedures were carried out as in Example 1 to detect a fusion protein. The results were shown in Fig. 14. In Fig. 14, the results of electrophoresis are shown as follows; M: molecular weight marker, lane 1: homogenate solution, lane 2: supernatant fraction after centrifugation, lane 3: precipitated fraction after centrifugation, lanes 4 to 7: flow-through fractions 1 to 4 of the ion exchange resin, lane 8: elution fraction by buffer A (NaCl concentration of 150 mM) (EL0), lane 9: elution fraction by a 3 : 1 mixed solution of buffer A : buffer B (NaCl concentration of 363 mM) (EL1), lane 10: elution fraction by a 1 : 1 mixed solution of buffer A : buffer B (NaCl concentration of 575 mM) (EL2), lane 11: elution fraction by a 1 : 3 mixed solution of buffer A : buffer B (NaCl concentration of 788 mM) (EL3), and lane 12: elution fraction by buffer B (NaCl concentration of 1000 mM) (EL4).

Comparative Example 1 [Expression and Purification of Fusion Protein DE6-HRV3Csite-NQO1 (pl = 6.4)]

[0077] Peptide tag DE6 was fused to the N-terminal side of NQO1 that is a target protein, and expression and purification of a fusion protein containing HRV3Csite between NQO1 and DE6 were performed.

[0078] The same procedures were carried out as in Example 1, except for preparing an insert part using polynucleotides (SEQ ID NOs.: 66 and 67) encoding DE6 (EEEDDD; SEQ ID NO.: 65) in place of the polynucleotides encoding the peptide tag DE12, to construct a pM01_DE6 vector.

[0079] Further, pM01_DE6_HRV3Csite_NQO1 was constructed, and a recombinant baculovirus was prepared in the same manner as in Example 1, then expression, purification and detection of fusion protein DE6-HRV3Csite-NQO1 were performed. The results were shown in Fig. 15.

(Regarding Results of Examples 1 to 13 and Comparative Example 1)

[0080] In Comparative Example 1 (DE6-HRV3Csite-NQO1, pl = 6.4), a fusion protein was contained in every fraction, and separation was insufficient. On the other hand, Examples 1 to 13 all showed good separation. Particularly, a fusion protein binding a peptide tag with 18 or more acidic amino acid residues was held in the ion exchange resin up to a high salt concentration of 500 mM or more, and separation from contaminant proteins was improved (Examples 2 to 13). Also in all three target proteins, good separation was obtained (Examples 6 to 7 and 11 to 13). The peptide tag, even the peptide tag containing both aspartic acid residue and glutamic acid residue as acidic amino acid residues, or the peptide tag only containing either one, equally showed good separation (Examples 3 and 8). Even when the binding position of the peptide tag is either N-terminal side or C-terminal side of the target protein, separation was good (Examples 6 to 7 and 9 to 10).

Example 14 [Separation and Detection of Target Protein NQ1 from Fusion Protein DE12-HRV3Csite-NQO1]

[0081] Protease DED-HRV3C was reacted with DE12-HRV3Csite-NQO1 to cleave DE12 from DE12-HRV3Csite-NQO1.

[0082] To Fraction EL2 collected in Example 1 was mixed a 1/10 volume of Fraction EL3 collected in Example 13, and the mixture was allowed to stand at 4°C. After 16 hours, 20 mM Tris-HCl (pH 8.0) was added to this reaction solution to be diluted to a NaCl concentration of about 250 mM, and the diluted solution was loaded on an ion exchange resin (Hitrap Q HP (1 mL)) equilibrated with 20 mM Tris-HCl (pH 8.0), 150 mM NaCl. Flow-through fractions were collected (Fr. 1 to 6 (NaCl concentration of about 250 mM)), and fractions finally eluted (Fr. 7 to 10) with only buffer B (NaCl concentration of about 1000 mM) were each collected.

[0083] SDS-PAGE was performed using 5 to 20% gradient gel and an electrophoresis apparatus (APRO Science Inc.). Each collected fraction and β-ME Sample Treatment for Tris SDS (COSMO BIO Co., Ltd.) were mixed in a volume ratio of 1:1, and treated at 100°C for 3 minutes, then 5 µL of the resulting solution was loaded on the gel. As a molecular weight marker, 2.5 µL of Blue Star (NIPPON Genetics Co., Ltd.) was loaded on the gel. The gel loaded with the sample

was migrated at a voltage of 400 V for 14 minutes, and then the gel was stained by GelCode Blue Stain Reagent (Thermo Fisher Scientific Inc.), and excess staining was bleached, then the image was photographed using Gel Doc XR+ system (Bio-Rad Laboratories, Inc.). The results were shown in Fig. 16. In Fig. 16, the results of electrophoresis are shown as follows; M: molecular weight marker, lane 1: EL2 (Examples 1 and 8) or EL3 (Examples 2 to 7 and 11 to 12), lane 2: EL3 of Example 13, lane 3: a mixed reaction liquid of EL2 or EL3 of Examples 1 to 12, and EL3 of Example 13, lanes 4 to 8: flow-through fractions 1 to 5 of the ion exchange resin, and lanes 9 to 13: elution fractions 1 to 5 by buffer B. Explanation of each lane is common in Figs. 17 to 25.

Example 15

[0084] The same procedures were carried out as in Example 14, except for using EL2 of Example 2 in place of EL2 of Example 1, to detect a target protein NQO1. The results were shown in Fig. 17.

Example 16

[0085] The same procedures were carried out as in Example 14, except for using EL3 of Example 3 in place of EL2 of Example 1, to detect a target protein NQO1. The results were shown in Fig. 18.

Example 17

[0086] The same procedures were carried out as in Example 14, except for using EL3 of Example 4 in place of EL2 of Example 1, to detect a target protein NQO1. The results were shown in Fig. 19.

Example 18

[0087] The same procedures were carried out as in Example 14, except for using EL3 of Example 5 in place of EL2 of Example 1, to detect a target protein NQO1. The results were shown in Fig. 20.

Example 19

[0088] The same procedures were carried out as in Example 14, except for using EL3 of Example 6 in place of EL2 of Example 1, to detect a target protein NQO1. The results were shown in Fig. 21.

Example 20

[0089] The same procedures were carried out as in Example 14, except for using EL3 of Example 7 in place of EL2 of Example 1, to detect a target protein NQO1. The results were shown in Fig. 22.

Example 21

[0090] The same procedures were carried out as in Example 14, except for using EL2 of Example 8 in place of EL2 of Example 1, to detect a target protein NQO1. The results were shown in Fig. 23.

Example 22

[0091] The same procedures were carried out as in Example 14, except for using EL3 of Example 11 in place of EL2 of Example 1, to detect a target protein Luciferase. The results were shown in Fig. 24.

Example 23

[0092] The same procedures were carried out as in Example 14, except for using EL3 of Example 12 in place of EL2 of Example 1, to detect a target protein Luciferase. The results were shown in Fig. 25.

(Regarding Results of Examples 14 to 23)

[0093] Based on the results of Examples 14 to 23, it was shown that the protease is reacted with the fusion protein, whereby a target protein NQO1 or Luciferase can be acquired in the flow-through fraction of the anion exchange resin.

Example 24 [Evaluation of Effect on Separation due to pH of Buffer]

[0094] The same procedures were carried out as in Example 6 to construct pHS01_DED_HRV3Csite_NQO1.

[0095] pHS01_DED_HRV3Csite_NQO1 and baculovirus DNA were introduced into silkworm culture cells (BmN cells), and homologous recombination of virus was performed. The culture cells were cultured for 6 days, and the recombinant virus was collected. Thereafter, a virus solution diluted 50 times with MilliQ water was inoculated into pupa of silkworm, and the pupa was incubated for 6 days. The pupa of silkworm was collected, and 50 mL of a buffer (20 mM PIPES (pH 6.1), 150 mM NaCl, 1 tablet Complete EDTA free (Roche), phenylthiourea) was added per a pupa to homogenate the pupa. The solution was centrifuged (8,000 g, 10 minutes), then the supernatant fraction was filtered with 0.8 μ M of a filter agent, and the filtrate was collected. The filtrate was loaded on an ion exchange resin (Hitrap Q HP (1 mL)), and purified using a mixed solution of 20 mM PIPES (pH 6.1, 150 mM NaCl; buffer C) and 20 mM PIPES (pH 6.1, 1000 mM NaCl; buffer D). First, only buffer C (NaCl concentration of 150 mM) was flown through the ion exchange resin (EL0), and a fraction eluted with a solution with a mixing ratio of buffer C : buffer D of 3 : 1 (NaCl concentration of 363 mM) (EL1), a fraction eluted with a solution with a mixing ratio of buffer C : buffer D of 1 : 1 (NaCl concentration of 575 mM) (EL2), a fraction eluted with a solution with a mixing ratio of buffer C : buffer D of 1 : 3 (NaCl concentration of 788 mM) (EL3), and finally, a fraction eluted with only buffer D (NaCl concentration of 1000 mM) (EL4) were each collected.

[0096] The same procedures were carried out as in Example 1 to detect DED-HRV3Csite-NQO1. The results were shown in Fig. 26. In Fig. 26, the results of electrophoresis are shown as follows; M: molecular weight marker, lane 1: homogenate solution, lane 2: supernatant fraction after centrifugation, lane 3: precipitated fraction after centrifugation, lanes 4 to 7: flow-through fractions 1 to 4 of the ion exchange resin, lane 8: elution fraction by buffer C (NaCl concentration of 150 mM) (EL0), lane 9: elution fraction by a 3 : 1 mixed solution of buffer C : buffer D (NaCl concentration of 363 mM) (EL1), lane 10: elution fraction by a 1 : 1 mixed solution of buffer C : buffer D (NaCl concentration of 575 mM) (EL2), lane 11: elution fraction by a 1 : 3 mixed solution of buffer C : buffer D (NaCl concentration of 788 mM) (EL3), and lane 12: elution fraction by buffer D (NaCl concentration of 1000 mM) (EL4). Explanation of each lane is common in Figs. 27 to 28.

Example 25

[0097] The same procedures were carried out as in Example 7 to construct pHS01_DES_HRV3Csite_NQO1.

[0098] Furthermore, the same procedures were carried out as in Example 24 to express, purify and detect DES-HRV3Csite-NQO1. The results were shown in Fig. 27.

Example 26

[0099] The same procedures were carried out as in Example 11 to construct pHS01_DED_HRV3Csite_Luciferase.

[0100] Furthermore, the same procedures were carried out as in Example 24 to express, purify and detect DED-HRV3Csite-Luciferase. The results were shown in Fig. 28.

Example 27

[0101] The same procedures were carried out as in Example 7 to construct pHS01_DES_HRV3Csite_NQO1.

[0102] E. coli was transformed using pHS01_DES_HRV3Csite_NQO1, and 1.5 L of an LB medium containing ampicillin was cultured at 37°C and cooled to 15°C around a turbidity OD of about 0.6, then IPTG was added thereto to induce expression of protein. After 16 hours, bacteria were collected by centrifugation (8,000 g, 15 minutes) and frozen at -80°C. 120 mL of a buffer (20 mM PIPES (pH 6.1), 150 mM NaCl, 1 tablet Complete EDTA free (Roche)) was added to the frozen E. coli, and E. coli was crushed by ultrasonic wave. The crushed solution was centrifuged (15,000 g x 30 minutes), then the supernatant fraction was filtered with 0.8 μ M of a filter agent, and the filtrate was collected. The filtrate was loaded on an ion exchange resin (Hitrap Q HP (1 mL)), and purified using a mixed solution of 20 mM PIPES (pH 6.1, 150 mM NaCl; buffer C) and 20 mM PIPES (pH 6.1, 1000 mM NaCl; buffer D). First, only buffer C (NaCl concentration of 150 mM) was flown through the ion exchange resin (EL0), and a fraction eluted with a 3 : 1 mixed solution of buffer C : buffer D (NaCl concentration of 363 mM) (EL1), a fraction eluted with a 1 : 1 mixed solution of buffer C : buffer D (NaCl concentration of 575 mM) (EL2), a fraction eluted with a 1 : 3 mixed solution of buffer C : buffer D (NaCl concentration of 788 mM) (EL3), and finally, a fraction eluted with only buffer D (NaCl concentration of 1000 mM) (EL4) were each collected.

[0103] The same procedures were carried out as in Example 1 to detect DED-HRV3Csite-NQO1. The results were shown in Fig. 29. In Fig. 29, the results of electrophoresis are shown as follows; M: molecular weight marker, lane 1: homogenate solution, lane 2: supernatant fraction after centrifugation, lane 3: precipitated fraction after centrifugation, lanes 4 to 7: flow-through fractions 1 to 4 of the ion exchange resin, lane 8: elution fraction by buffer C (NaCl concentration

of 150 mM) (EL0), lane 9: elution fraction by a 3 : 1 mixed solution of buffer C : buffer D (NaCl concentration of 363 mM) (EL1), lane 10: elution fraction by a 1 : 1 mixed solution of buffer C : buffer D (NaCl concentration of 575 mM) (EL2), lane 11: elution fraction by a 1 : 3 mixed solution of buffer C : buffer D (NaCl concentration of 788 mM) (EL3), and lane 12: elution fraction by buffer D (NaCl concentration of 1000 mM; EL4).

Example 28

[0104] To EL3 of Example 24 was added a 1/10 volume of EL3 of Example 27, and the mixture was allowed to stand at 4°C. After 16 hours, 20 mM PIPES (pH 6.1) was added to the reaction solution to be diluted to a NaCl concentration of about 250 mM, and the diluted solution was loaded on an ion exchange resin (Hitrap Q HP (1 mL)) equilibrated with 20 mM PIPES (pH 6.1, 150 mM NaCl). Flow-through fractions were collected (Fr. 1 to 6 (NaCl concentration of about 250 mM)), and fractions finally eluted (Fr. 7 to 10) with only buffer D (NaCl concentration of about 1000 mM) were each collected.

[0105] The same procedures were carried out as in Example 1 to detect a target protein EQO1. The results were shown in Fig. 30. In Fig. 30, the results of electrophoresis are shown as follows; M: molecular weight marker, lane 1: EL3 of Example 24, lane 2: EL3 of Example 27, lane 3: a mixed reaction liquid of EL3 of Example 24, and EL3 of Example 27, lanes 4 to 8: flow-through fractions 1 to 5 of the ion exchange resin, and lanes 9 to 13: elution fractions 1 to 5 by buffer D.

(Regarding Results of Examples 24 to 28)

[0106] Based on the results of Examples 24 to 28, it was shown that, in the expression and purification of the fusion protein and purification of the target protein, good separation is obtained even the pH of the buffer is lowered.

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	Leu	Glu	Val	Leu	Phe	Gln	Gly	Pro	Met	Val	Gly	Arg	Arg	Ala	Leu	Ile	20	25	30	
	Val	Leu	Ala	His	Ser	Glu	Arg	Thr	Ser	Phe	Asn	Tyr	Ala	Met	Lys	Glu	35	40	45	
	Ala	Ala	Ala	Ala	Ala	Leu	Lys	Lys	Lys	Gly	Trp	Glu	Val	Val	Glu	Ser	50	55	60	
	Asp	Leu	Tyr	Ala	Met	Asn	Phe	Asn	Pro	Ile	Ile	Ser	Arg	Lys	Asp	Ile	65	70	75	80

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	Thr	Gly	Lys	Leu	Lys	Asp	Pro	Ala	Asn	Phe	Gln	Tyr	Pro	Ala	Glu	Ser	
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5	Val	Leu	Ala	Tyr	Lys	Glu	Gly	His	Leu	Ser	Pro	Asp	Ile	Val	Ala	Glu	
				100					105					110			
	Gln	Lys	Lys	Leu	Glu	Ala	Ala	Asp	Leu	Val	Ile	Phe	Gln	Phe	Pro	Leu	
10			115					120					125				
	Gln	Trp	Phe	Gly	Val	Pro	Ala	Ile	Leu	Lys	Gly	Trp	Phe	Glu	Arg	Val	
		130					135					140					
15	Phe	Ile	Gly	Glu	Phe	Ala	Tyr	Thr	Tyr	Ala	Ala	Met	Tyr	Asp	Lys	Gly	
	145					150					155					160	
	Pro	Phe	Arg	Ser	Lys	Lys	Ala	Val	Leu	Ser	Ile	Thr	Thr	Gly	Gly	Ser	
20					165					170					175		
	Gly	Ser	Met	Tyr	Ser	Leu	Gln	Gly	Ile	His	Gly	Asp	Met	Asn	Val	Ile	
				180					185					190			
25	Leu	Trp	Pro	Ile	Gln	Ser	Gly	Ile	Leu	His	Phe	Cys	Gly	Phe	Gln	Val	
			195					200					205				
	Leu	Glu	Pro	Gln	Leu	Thr	Tyr	Ser	Ile	Gly	His	Thr	Pro	Ala	Asp	Ala	
30		210					215					220					
	Arg	Ile	Gln	Ile	Leu	Glu	Gly	Trp	Lys	Lys	Arg	Leu	Glu	Asn	Ile	Trp	
35	225				230						235					240	
	Asp	Glu	Thr	Pro	Leu	Tyr	Phe	Ala	Pro	Ser	Ser	Leu	Phe	Asp	Leu	Asn	
					245					250					255		
40	Phe	Gln	Ala	Gly	Phe	Leu	Met	Lys	Lys	Glu	Val	Gln	Asp	Glu	Glu	Lys	
				260					265					270			
	Asn	Lys	Lys	Phe	Gly	Leu	Ser	Val	Gly	His	His	Leu	Gly	Lys	Ser	Ile	
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10	Asp	Asp	Asp	Lys	Gly	Thr	Leu	Glu	Val	Leu	Phe	Gln	Gly	Pro	Met	Val	20	25	30		
15	Gly	Arg	Arg	Ala	Leu	Ile	Val	Leu	Ala	His	Ser	Glu	Arg	Thr	Ser	Phe	35	40	45		
20	Asn	Tyr	Ala	Met	Lys	Glu	Ala	Ala	Ala	Ala	Ala	Leu	Lys	Lys	Lys	Gly	50	55	60		
25	Trp	Glu	Val	Val	Glu	Ser	Asp	Leu	Tyr	Ala	Met	Asn	Phe	Asn	Pro	Ile	65	70	75	80	
30	Ile	Ser	Arg	Lys	Asp	Ile	Thr	Gly	Lys	Leu	Lys	Asp	Pro	Ala	Asn	Phe	85	90	95		
35	Gln	Tyr	Pro	Ala	Glu	Ser	Val	Leu	Ala	Tyr	Lys	Glu	Gly	His	Leu	Ser	100	105	110		
40	Pro	Asp	Ile	Val	Ala	Glu	Gln	Lys	Lys	Leu	Glu	Ala	Ala	Asp	Leu	Val	115	120	125		
45	Ile	Phe	Gln	Phe	Pro	Leu	Gln	Trp	Phe	Gly	Val	Pro	Ala	Ile	Leu	Lys	130	135	140		
50	Gly	Trp	Phe	Glu	Arg	Val	Phe	Ile	Gly	Glu	Phe	Ala	Tyr	Thr	Tyr	Ala	145	150	155	160	
55	Ala	Met	Tyr	Asp	Lys	Gly	Pro	Phe	Arg	Ser	Lys	Lys	Ala	Val	Leu	Ser	165	170	175		
	Ile	Thr	Thr	Gly	Gly	Ser	Gly	Ser	Met	Tyr	Ser	Leu	Gln	Gly	Ile	His	180	185	190		
	Gly	Asp	Met	Asn	Val	Ile	Leu	Trp	Pro	Ile	Gln	Ser	Gly	Ile	Leu	His	195	200	205		
	Phe	Cys	Gly	Phe	Gln	Val	Leu	Glu	Pro	Gln	Leu	Thr	Tyr	Ser	Ile	Gly	210	215	220		
	His	Thr	Pro	Ala	Asp	Ala	Arg	Ile	Gln	Ile	Leu	Glu	Gly	Trp	Lys	Lys	225	230	235	240	

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	Arg	Leu	Glu	Asn	Ile	Trp	Asp	Glu	Thr	Pro	Leu	Tyr	Phe	Ala	Pro	Ser	
					245					250					255		
5	Ser	Leu	Phe	Asp	Leu	Asn	Phe	Gln	Ala	Gly	Phe	Leu	Met	Lys	Lys	Glu	
				260					265					270			
	Val	Gln	Asp	Glu	Glu	Lys	Asn	Lys	Lys	Phe	Gly	Leu	Ser	Val	Gly	His	
10			275					280					285				
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30				20					25					30			
	Phe	Gln	Gly	Pro	Met	Val	Gly	Arg	Arg	Ala	Leu	Ile	Val	Leu	Ala	His	
			35					40					45				
35	Ser	Glu	Arg	Thr	Ser	Phe	Asn	Tyr	Ala	Met	Lys	Glu	Ala	Ala	Ala	Ala	
		50					55					60					
	Ala	Leu	Lys	Lys	Lys	Gly	Trp	Glu	Val	Val	Glu	Ser	Asp	Leu	Tyr	Ala	
40	65					70					75					80	
	Met	Asn	Phe	Asn	Pro	Ile	Ile	Ser	Arg	Lys	Asp	Ile	Thr	Gly	Lys	Leu	
				85						90					95		
45	Lys	Asp	Pro	Ala	Asn	Phe	Gln	Tyr	Pro	Ala	Glu	Ser	Val	Leu	Ala	Tyr	
				100					105					110			
	Lys	Glu	Gly	His	Leu	Ser	Pro	Asp	Ile	Val	Ala	Glu	Gln	Lys	Lys	Leu	
50			115					120					125				
	Glu	Ala	Ala	Asp	Leu	Val	Ile	Phe	Gln	Phe	Pro	Leu	Gln	Trp	Phe	Gly	
		130					135					140					
55	Val	Pro	Ala	Ile	Leu	Lys	Gly	Trp	Phe	Glu	Arg	Val	Phe	Ile	Gly	Glu	

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	145		150		155		160									
5	Phe	Ala	Tyr	Thr	Tyr	Ala	Ala	Met	Tyr	Asp	Lys	Gly	Pro	Phe	Arg	Ser
					165					170					175	
10	Lys	Lys	Ala	Val	Leu	Ser	Ile	Thr	Thr	Gly	Gly	Ser	Gly	Ser	Met	Tyr
				180						185				190		
15	Ser	Leu	Gln	Gly	Ile	His	Gly	Asp	Met	Asn	Val	Ile	Leu	Trp	Pro	Ile
			195					200					205			
20	Gln	Ser	Gly	Ile	Leu	His	Phe	Cys	Gly	Phe	Gln	Val	Leu	Glu	Pro	Gln
		210					215					220				
25	Leu	Thr	Tyr	Ser	Ile	Gly	His	Thr	Pro	Ala	Asp	Ala	Arg	Ile	Gln	Ile
	225					230					235				240	
30	Leu	Glu	Gly	Trp	Lys	Lys	Arg	Leu	Glu	Asn	Ile	Trp	Asp	Glu	Thr	Pro
					245					250				255		
35	Leu	Tyr	Phe	Ala	Pro	Ser	Ser	Leu	Phe	Asp	Leu	Asn	Phe	Gln	Ala	Gly
				260					265					270		
40	Phe	Leu	Met	Lys	Lys	Glu	Val	Gln	Asp	Glu	Glu	Lys	Asn	Lys	Lys	Phe
			275					280					285			
45	Gly	Leu	Ser	Val	Gly	His	His	Leu	Gly	Lys	Ser	Ile	Pro	Thr	Asp	Asn
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				20					25					30		
70	Gly	Thr	Leu	Glu	Val	Leu	Phe	Gln	Gly	Pro	Met	Val	Gly	Arg	Arg	Ala
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	Leu	Ile	Val	Leu	Ala	His	Ser	Glu	Arg	Thr	Ser	Phe	Asn	Tyr	Ala	Met	
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5	Lys	Glu	Ala	Ala	Ala	Ala	Ala	Leu	Lys	Lys	Lys	Gly	Trp	Glu	Val	Val	
	65					70					75					80	
	Glu	Ser	Asp	Leu	Tyr	Ala	Met	Asn	Phe	Asn	Pro	Ile	Ile	Ser	Arg	Lys	
10				85						90					95		
	Asp	Ile	Thr	Gly	Lys	Leu	Lys	Asp	Pro	Ala	Asn	Phe	Gln	Tyr	Pro	Ala	
				100					105					110			
15																	
	Glu	Ser	Val	Leu	Ala	Tyr	Lys	Glu	Gly	His	Leu	Ser	Pro	Asp	Ile	Val	
			115					120					125				
20	Ala	Glu	Gln	Lys	Lys	Leu	Glu	Ala	Ala	Asp	Leu	Val	Ile	Phe	Gln	Phe	
	130					135						140					
	Pro	Leu	Gln	Trp	Phe	Gly	Val	Pro	Ala	Ile	Leu	Lys	Gly	Trp	Phe	Glu	
25	145				150						155					160	
	Arg	Val	Phe	Ile	Gly	Glu	Phe	Ala	Tyr	Thr	Tyr	Ala	Ala	Met	Tyr	Asp	
				165						170					175		
30	Lys	Gly	Pro	Phe	Arg	Ser	Lys	Lys	Ala	Val	Leu	Ser	Ile	Thr	Thr	Gly	
				180					185					190			
	Gly	Ser	Gly	Ser	Met	Tyr	Ser	Leu	Gln	Gly	Ile	His	Gly	Asp	Met	Asn	
35			195					200					205				
	Val	Ile	Leu	Trp	Pro	Ile	Gln	Ser	Gly	Ile	Leu	His	Phe	Cys	Gly	Phe	
40			210				215					220					
	Gln	Val	Leu	Glu	Pro	Gln	Leu	Thr	Tyr	Ser	Ile	Gly	His	Thr	Pro	Ala	
	225				230						235					240	
45	Asp	Ala	Arg	Ile	Gln	Ile	Leu	Glu	Gly	Trp	Lys	Lys	Arg	Leu	Glu	Asn	
				245						250					255		
	Ile	Trp	Asp	Glu	Thr	Pro	Leu	Tyr	Phe	Ala	Pro	Ser	Ser	Leu	Phe	Asp	
50				260					265					270			
	Leu	Asn	Phe	Gln	Ala	Gly	Phe	Leu	Met	Lys	Lys	Glu	Val	Gln	Asp	Glu	
		275				280						285					
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		35	40 45
	Met Val Gly Arg Arg Ala Leu Ile Val Leu Ala His Ser Glu Arg Thr		
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30	Ser Phe Asn Tyr Ala Met Lys Glu Ala Ala Ala Ala Ala Leu Lys Lys		
		65	70 75 80
35	Lys Gly Trp Glu Val Val Glu Ser Asp Leu Tyr Ala Met Asn Phe Asn		
		85	90 95
40	Pro Ile Ile Ser Arg Lys Asp Ile Thr Gly Lys Leu Lys Asp Pro Ala		
		100	105 110
	Asn Phe Gln Tyr Pro Ala Glu Ser Val Leu Ala Tyr Lys Glu Gly His		
		115	120 125
45	Leu Ser Pro Asp Ile Val Ala Glu Gln Lys Lys Leu Glu Ala Ala Asp		
		130	135 140
50	Leu Val Ile Phe Gln Phe Pro Leu Gln Trp Phe Gly Val Pro Ala Ile		
		145	150 155 160
	Leu Lys Gly Trp Phe Glu Arg Val Phe Ile Gly Glu Phe Ala Tyr Thr		
		165	170 175
55	Tyr Ala Ala Met Tyr Asp Lys Gly Pro Phe Arg Ser Lys Lys Ala Val		
		180	185 190

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Leu Ser Ile Thr Thr Gly Gly Ser Gly Ser Met Tyr Ser Leu Gln Gly
195 200 205

5 Ile His Gly Asp Met Asn Val Ile Leu Trp Pro Ile Gln Ser Gly Ile
210 215 220

10 Leu His Phe Cys Gly Phe Gln Val Leu Glu Pro Gln Leu Thr Tyr Ser
225 230 235 240

Ile Gly His Thr Pro Ala Asp Ala Arg Ile Gln Ile Leu Glu Gly Trp
245 250 255

15 Lys Lys Arg Leu Glu Asn Ile Trp Asp Glu Thr Pro Leu Tyr Phe Ala
260 265 270

20 Pro Ser Ser Leu Phe Asp Leu Asn Phe Gln Ala Gly Phe Leu Met Lys
275 280 285

25 Lys Glu Val Gln Asp Glu Glu Lys Asn Lys Lys Phe Gly Leu Ser Val
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Gly His His Leu Gly Lys Ser Ile Pro Thr Asp Asn Gln Ile Lys Ala
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30 Arg Lys

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35 40 45

55 Asp Asp Asp Glu Asp Ser Gly Ala Glu Ile Gln Asp Asp Asp Glu Glu
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	Gly	Phe	Asp	Asp	Glu	Glu	Glu	Phe	Asp	Asp	Asp	Asp	Asp	Asp	Glu	His	
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5	Asp	Asp	Asp	Asp	Leu	Glu	Asn	Glu	Glu	Asn	Glu	Leu	Glu	Glu	Leu	Glu	
					85					90						95	
10	Glu	Arg	Val	Glu	Ala	Arg	Lys	Lys	Ala	Ser	Leu	Glu	Val	Leu	Phe	Gln	
				100					105						110		
15	Gly	Pro	Met	Val	Gly	Arg	Arg	Ala	Leu	Ile	Val	Leu	Ala	His	Ser	Glu	
			115					120					125				
20	Arg	Thr	Ser	Phe	Asn	Tyr	Ala	Met	Lys	Glu	Ala	Ala	Ala	Ala	Ala	Leu	
		130					135					140					
25	Lys	Lys	Lys	Gly	Trp	Glu	Val	Val	Glu	Ser	Asp	Leu	Tyr	Ala	Met	Asn	
	145					150					155					160	
30	Phe	Asn	Pro	Ile	Ile	Ser	Arg	Lys	Asp	Ile	Thr	Gly	Lys	Leu	Lys	Asp	
				165						170					175		
35	Pro	Ala	Asn	Phe	Gln	Tyr	Pro	Ala	Glu	Ser	Val	Leu	Ala	Tyr	Lys	Glu	
			180						185					190			
40	Gly	His	Leu	Ser	Pro	Asp	Ile	Val	Ala	Glu	Gln	Lys	Lys	Leu	Glu	Ala	
			195					200					205				
45	Ala	Asp	Leu	Val	Ile	Phe	Gln	Phe	Pro	Leu	Gln	Trp	Phe	Gly	Val	Pro	
		210					215					220					
50	Ala	Ile	Leu	Lys	Gly	Trp	Phe	Glu	Arg	Val	Phe	Ile	Gly	Glu	Phe	Ala	
	225				230						235					240	
55	Tyr	Thr	Tyr	Ala	Ala	Met	Tyr	Asp	Lys	Gly	Pro	Phe	Arg	Ser	Lys	Lys	
				245						250					255		
60	Ala	Val	Leu	Ser	Ile	Thr	Thr	Gly	Gly	Ser	Gly	Ser	Met	Tyr	Ser	Leu	
			260						265					270			
65	Gln	Gly	Ile	His	Gly	Asp	Met	Asn	Val	Ile	Leu	Trp	Pro	Ile	Gln	Ser	
		275						280					285				
70	Gly	Ile	Leu	His	Phe	Cys	Gly	Phe	Gln	Val	Leu	Glu	Pro	Gln	Leu	Thr	
		290					295					300					
75	Tyr	Ser	Ile	Gly	His	Thr	Pro	Ala	Asp	Ala	Arg	Ile	Gln	Ile	Leu	Glu	
	305					310					315					320	

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Gly Trp Lys Lys Arg Leu Glu Asn Ile Trp Asp Glu Thr Pro Leu Tyr
325 330 335

5 Phe Ala Pro Ser Ser Leu Phe Asp Leu Asn Phe Gln Ala Gly Phe Leu
340 345 350

10 Met Lys Lys Glu Val Gln Asp Glu Glu Lys Asn Lys Lys Phe Gly Leu
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Ser Val Gly His His Leu Gly Lys Ser Ile Pro Thr Asp Asn Gln Ile
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15 Lys Ala Arg Lys
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35 40 45

40 Asp Asp Asp Glu Asp Ser Gly Ala Glu Ala Ser Leu Glu Val Leu Phe
50 55 60

45 Gln Gly Pro Met Val Gly Arg Arg Ala Leu Ile Val Leu Ala His Ser
65 70 75 80

Glu Arg Thr Ser Phe Asn Tyr Ala Met Lys Glu Ala Ala Ala Ala Ala
85 90 95

50 Leu Lys Lys Lys Gly Trp Glu Val Val Glu Ser Asp Leu Tyr Ala Met
100 105 110

55 Asn Phe Asn Pro Ile Ile Ser Arg Lys Asp Ile Thr Gly Lys Leu Lys
115 120 125

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5	Glu	Gly	His	Leu	Ser	Pro	Asp	Ile	Val	Ala	Glu	Gln	Lys	Lys	Leu	Glu	
	145					150					155					160	
	Ala	Ala	Asp	Leu	Val	Ile	Phe	Gln	Phe	Pro	Leu	Gln	Trp	Phe	Gly	Val	
10				165						170					175		
	Pro	Ala	Ile	Leu	Lys	Gly	Trp	Phe	Glu	Arg	Val	Phe	Ile	Gly	Glu	Phe	
				180					185					190			
15	Ala	Tyr	Thr	Tyr	Ala	Ala	Met	Tyr	Asp	Lys	Gly	Pro	Phe	Arg	Ser	Lys	
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	Lys	Ala	Val	Leu	Ser	Ile	Thr	Thr	Gly	Gly	Ser	Gly	Ser	Met	Tyr	Ser	
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	Leu	Gln	Gly	Ile	His	Gly	Asp	Met	Asn	Val	Ile	Leu	Trp	Pro	Ile	Gln	
25	225					230					235					240	
	Ser	Gly	Ile	Leu	His	Phe	Cys	Gly	Phe	Gln	Val	Leu	Glu	Pro	Gln	Leu	
				245						250					255		
30	Thr	Tyr	Ser	Ile	Gly	His	Thr	Pro	Ala	Asp	Ala	Arg	Ile	Gln	Ile	Leu	
				260					265					270			
	Glu	Gly	Trp	Lys	Lys	Arg	Leu	Glu	Asn	Ile	Trp	Asp	Glu	Thr	Pro	Leu	
35			275					280					285				
	Tyr	Phe	Ala	Pro	Ser	Ser	Leu	Phe	Asp	Leu	Asn	Phe	Gln	Ala	Gly	Phe	
		290					295					300					
40	Leu	Met	Lys	Lys	Glu	Val	Gln	Asp	Glu	Glu	Lys	Asn	Lys	Lys	Phe	Gly	
	305					310					315					320	
	Leu	Ser	Val	Gly	His	His	Leu	Gly	Lys	Ser	Ile	Pro	Thr	Asp	Asn	Gln	
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15	Glu	Glu	Glu	Glu	Glu	Ala	Ser	Leu	Glu	Val	Leu	Phe	Gln	Gly	Pro	Met	35	40	45	
20	Val	Gly	Arg	Arg	Ala	Leu	Ile	Val	Leu	Ala	His	Ser	Glu	Arg	Thr	Ser	50	55	60	
25	Phe	Asn	Tyr	Ala	Met	Lys	Glu	Ala	Ala	Ala	Ala	Ala	Leu	Lys	Lys	Lys	65	70	75	80
30	Gly	Trp	Glu	Val	Val	Glu	Ser	Asp	Leu	Tyr	Ala	Met	Asn	Phe	Asn	Pro	85	90	95	
35	Ile	Ile	Ser	Arg	Lys	Asp	Ile	Thr	Gly	Lys	Leu	Lys	Asp	Pro	Ala	Asn	100	105	110	
40	Phe	Gln	Tyr	Pro	Ala	Glu	Ser	Val	Leu	Ala	Tyr	Lys	Glu	Gly	His	Leu	115	120	125	
45	Ser	Pro	Asp	Ile	Val	Ala	Glu	Gln	Lys	Lys	Leu	Glu	Ala	Ala	Asp	Leu	130	135	140	
50	Val	Ile	Phe	Gln	Phe	Pro	Leu	Gln	Trp	Phe	Gly	Val	Pro	Ala	Ile	Leu	145	150	155	160
55	Lys	Gly	Trp	Phe	Glu	Arg	Val	Phe	Ile	Gly	Glu	Phe	Ala	Tyr	Thr	Tyr	165	170	175	
	Ala	Ala	Met	Tyr	Asp	Lys	Gly	Pro	Phe	Arg	Ser	Lys	Lys	Ala	Val	Leu	180	185	190	
	Ser	Ile	Thr	Thr	Gly	Gly	Ser	Gly	Ser	Met	Tyr	Ser	Leu	Gln	Gly	Ile	195	200	205	
	His	Gly	Asp	Met	Asn	Val	Ile	Leu	Trp	Pro	Ile	Gln	Ser	Gly	Ile	Leu	210	215	220	
	His	Phe	Cys	Gly	Phe	Gln	Val	Leu	Glu	Pro	Gln	Leu	Thr	Tyr	Ser	Ile	225	230	235	240

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	Gly	His	Thr	Pro	Ala	Asp	Ala	Arg	Ile	Gln	Ile	Leu	Glu	Gly	Trp	Lys	
					245					250					255		
5	Lys	Arg	Leu	Glu	Asn	Ile	Trp	Asp	Glu	Thr	Pro	Leu	Tyr	Phe	Ala	Pro	
				260					265					270			
10	Ser	Ser	Leu	Phe	Asp	Leu	Asn	Phe	Gln	Ala	Gly	Phe	Leu	Met	Lys	Lys	
			275					280					285				
15	Glu	Val	Gln	Asp	Glu	Glu	Lys	Asn	Lys	Lys	Phe	Gly	Leu	Ser	Val	Gly	
		290					295					300					
20	His	His	Leu	Gly	Lys	Ser	Ile	Pro	Thr	Asp	Asn	Gln	Ile	Lys	Ala	Arg	
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			20					25						30			
45	Lys	Gly	Trp	Glu	Val	Val	Glu	Ser	Asp	Leu	Tyr	Ala	Met	Asn	Phe	Asn	
		35					40					45					
50	Pro	Ile	Ile	Ser	Arg	Lys	Asp	Ile	Thr	Gly	Lys	Leu	Lys	Asp	Pro	Ala	
		50				55						60					
55	Asn	Phe	Gln	Tyr	Pro	Ala	Glu	Ser	Val	Leu	Ala	Tyr	Lys	Glu	Gly	His	
	65				70					75						80	
60	Leu	Ser	Pro	Asp	Ile	Val	Ala	Glu	Gln	Lys	Lys	Leu	Glu	Ala	Ala	Asp	
				85					90					95			
65	Leu	Val	Ile	Phe	Gln	Phe	Pro	Leu	Gln	Trp	Phe	Gly	Val	Pro	Ala	Ile	
			100						105					110			

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			115					120					125				
5	Tyr	Ala	Ala	Met	Tyr	Asp	Lys	Gly	Pro	Phe	Arg	Ser	Lys	Lys	Ala	Val	
		130					135					140					
10	Leu	Ser	Ile	Thr	Thr	Gly	Gly	Ser	Gly	Ser	Met	Tyr	Ser	Leu	Gln	Gly	
	145					150					155				160		
15	Ile	His	Gly	Asp	Met	Asn	Val	Ile	Leu	Trp	Pro	Ile	Gln	Ser	Gly	Ile	
				165						170					175		
20	Leu	His	Phe	Cys	Gly	Phe	Gln	Val	Leu	Glu	Pro	Gln	Leu	Thr	Tyr	Ser	
				180					185					190			
25	Ile	Gly	His	Thr	Pro	Ala	Asp	Ala	Arg	Ile	Gln	Ile	Leu	Glu	Gly	Trp	
			195					200					205				
30	Lys	Lys	Arg	Leu	Glu	Asn	Ile	Trp	Asp	Glu	Thr	Pro	Leu	Tyr	Phe	Ala	
		210					215					220					
35	Pro	Ser	Ser	Leu	Phe	Asp	Leu	Asn	Phe	Gln	Ala	Gly	Phe	Leu	Met	Lys	
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40	Lys	Glu	Val	Gln	Asp	Glu	Glu	Lys	Asn	Lys	Lys	Phe	Gly	Leu	Ser	Val	
				245						250					255		
45	Gly	His	His	Leu	Gly	Lys	Ser	Ile	Pro	Thr	Asp	Asn	Gln	Ile	Lys	Ala	
				260					265					270			
50	Arg	Lys	Gly	Leu	Glu	Val	Leu	Phe	Gln	Gly	Pro	Ala	Ser	Asp	Leu	Ser	
			275					280					285				
55	Asn	Val	Glu	Gly	Lys	Thr	Gly	Asn	Ala	Thr	Asp	Glu	Glu	Glu	Glu	Glu	
	290						295					300					
60	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	
	305					310					315				320		
65	Asp	Asp	Asp	Glu	Asp	Ser	Gly	Ala	Glu	Ile	Gln	Asp	Asp	Asp	Glu	Glu	
				325						330					335		
70	Gly	Phe	Asp	Asp	Glu	Glu	Glu	Phe	Asp	Asp	Asp	Asp	Asp	Asp	Glu	His	
			340						345					350			
75	Asp	Asp	Asp	Asp	Leu	Glu	Asn	Glu	Glu	Asn	Glu	Leu	Glu	Glu	Leu	Glu	
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Asp Lys
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35 40 45

Pro Ile Ile Ser Arg Lys Asp Ile Thr Gly Lys Leu Lys Asp Pro Ala
50 55 60

Asn Phe Gln Tyr Pro Ala Glu Ser Val Leu Ala Tyr Lys Glu Gly His
65 70 75 80

Leu Ser Pro Asp Ile Val Ala Glu Gln Lys Lys Leu Glu Ala Ala Asp
85 90 95

Leu Val Ile Phe Gln Phe Pro Leu Gln Trp Phe Gly Val Pro Ala Ile
100 105 110

Leu Lys Gly Trp Phe Glu Arg Val Phe Ile Gly Glu Phe Ala Tyr Thr
115 120 125

Tyr Ala Ala Met Tyr Asp Lys Gly Pro Phe Arg Ser Lys Lys Ala Val
130 135 140

Leu Ser Ile Thr Thr Gly Gly Ser Gly Ser Met Tyr Ser Leu Gln Gly
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Ile His Gly Asp Met Asn Val Ile Leu Trp Pro Ile Gln Ser Gly Ile
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	Leu	His	Phe	Cys	Gly	Phe	Gln	Val	Leu	Glu	Pro	Gln	Leu	Thr	Tyr	Ser	
				180					185					190			
5	Ile	Gly	His	Thr	Pro	Ala	Asp	Ala	Arg	Ile	Gln	Ile	Leu	Glu	Gly	Trp	
			195					200					205				
	Lys	Lys	Arg	Leu	Glu	Asn	Ile	Trp	Asp	Glu	Thr	Pro	Leu	Tyr	Phe	Ala	
10		210					215					220					
	Pro	Ser	Ser	Leu	Phe	Asp	Leu	Asn	Phe	Gln	Ala	Gly	Phe	Leu	Met	Lys	
	225					230					235					240	
15	Lys	Glu	Val	Gln	Asp	Glu	Glu	Lys	Asn	Lys	Lys	Phe	Gly	Leu	Ser	Val	
					245					250					255		
	Gly	His	His	Leu	Gly	Lys	Ser	Ile	Pro	Thr	Asp	Asn	Gln	Ile	Lys	Ala	
20				260					265					270			
	Arg	Lys	Gly	Leu	Glu	Val	Leu	Phe	Gln	Gly	Pro	Ala	Ser	Asp	Leu	Ser	
			275					280					285				
25	Asn	Val	Glu	Gly	Lys	Thr	Gly	Asn	Ala	Thr	Asp	Glu	Glu	Glu	Glu	Glu	
		290					295					300					
	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	
30	305					310					315					320	
	Asp	Asp	Asp	Glu	Asp	Ser	Gly	Ala	Glu	Ala	Ser	Asp	Tyr	Lys	Asp	Asp	
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				20					25					30			
55	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	

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	35	40	45
5	Asp Asp Asp Glu Asp Ser Gly Ala Glu Ile Gln Asp Asp Asp Glu Glu 50 55 60		
10	Gly Phe Asp Asp Glu Glu Glu Phe Asp Asp Asp Asp Asp Asp Glu His 65 70 75 80		
15	Asp Asp Asp Asp Leu Glu Asn Glu Glu Asn Glu Leu Glu Glu Leu Glu 85 90 95		
20	Glu Arg Val Glu Ala Arg Lys Lys Ala Ser Leu Glu Val Leu Phe Gln 100 105 110		
25	Gly Pro Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr 115 120 125		
30	Pro Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys 130 135 140		
35	Arg Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile 145 150 155 160		
40	Glu Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu 165 170 175		
45	Ala Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val 180 185 190		
50	Val Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala 195 200 205		
55	Leu Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu 210 215 220		
	Arg Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe 225 230 235 240		
	Val Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu 245 250 255		
	Pro Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln 260 265 270		
	Gly Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly 275 280 285		

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	Phe	Asn	Glu	Tyr	Asp	Phe	Val	Pro	Glu	Ser	Phe	Asp	Arg	Asp	Lys	Thr	
	290						295					300					
5	Ile	Ala	Leu	Ile	Met	Asn	Ser	Ser	Gly	Ser	Thr	Gly	Leu	Pro	Lys	Gly	
	305					310					315					320	
	Val	Ala	Leu	Pro	His	Arg	Thr	Ala	Cys	Val	Arg	Phe	Ser	His	Ala	Arg	
10					325					330					335		
	Asp	Pro	Ile	Phe	Gly	Asn	Gln	Ile	Ile	Pro	Asp	Thr	Ala	Ile	Leu	Ser	
				340					345					350			
15	Val	Val	Pro	Phe	His	His	Gly	Phe	Gly	Met	Phe	Thr	Thr	Leu	Gly	Tyr	
			355					360					365				
	Leu	Ile	Cys	Gly	Phe	Arg	Val	Val	Leu	Met	Tyr	Arg	Phe	Glu	Glu	Glu	
20		370					375					380					
	Leu	Phe	Leu	Arg	Ser	Leu	Gln	Asp	Tyr	Lys	Ile	Gln	Ser	Ala	Leu	Leu	
25	385					390					395					400	
	Val	Pro	Thr	Leu	Phe	Ser	Phe	Phe	Ala	Lys	Ser	Thr	Leu	Ile	Asp	Lys	
					405					410					415		
30	Tyr	Asp	Leu	Ser	Asn	Leu	His	Glu	Ile	Ala	Ser	Gly	Gly	Ala	Pro	Leu	
				420					425					430			
	Ser	Lys	Glu	Val	Gly	Glu	Ala	Val	Ala	Lys	Arg	Phe	His	Leu	Pro	Gly	
35			435					440					445				
	Ile	Arg	Gln	Gly	Tyr	Gly	Leu	Thr	Glu	Thr	Thr	Ser	Ala	Ile	Leu	Ile	
	450						455					460					
40	Thr	Pro	Glu	Gly	Asp	Asp	Lys	Pro	Gly	Ala	Val	Gly	Lys	Val	Val	Pro	
	465					470					475					480	
	Phe	Phe	Glu	Ala	Lys	Val	Val	Asp	Leu	Asp	Thr	Gly	Lys	Thr	Leu	Gly	
45					485					490					495		
	Val	Asn	Gln	Arg	Gly	Glu	Leu	Cys	Val	Arg	Gly	Pro	Met	Ile	Met	Ser	
50				500					505					510			
	Gly	Tyr	Val	Asn	Asn	Pro	Glu	Ala	Thr	Asn	Ala	Leu	Ile	Asp	Lys	Asp	
			515					520					525				
55	Gly	Trp	Leu	His	Ser	Gly	Asp	Ile	Ala	Tyr	Trp	Asp	Glu	Asp	Glu	His	
	530						535					540					

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	Phe	Phe	Ile	Val	Asp	Arg	Leu	Lys	Ser	Leu	Ile	Lys	Tyr	Lys	Gly	Tyr	545	550	555				560
5	Gln	Val	Ala	Pro	Ala	Glu	Leu	Glu	Ser	Ile	Leu	Leu	Gln	His	Pro	Asn		565	570			575	
10	Ile	Phe	Asp	Ala	Gly	Val	Ala	Gly	Leu	Pro	Asp	Asp	Asp	Ala	Gly	Glu		580	585			590	
	Leu	Pro	Ala	Ala	Val	Val	Val	Leu	Glu	His	Gly	Lys	Thr	Met	Thr	Glu		595	600			605	
15	Lys	Glu	Ile	Val	Asp	Tyr	Val	Ala	Ser	Gln	Val	Thr	Thr	Ala	Lys	Lys		610	615			620	
20	Leu	Arg	Gly	Gly	Val	Val	Phe	Val	Asp	Glu	Val	Pro	Lys	Gly	Leu	Thr		625	630			635	
25	Gly	Lys	Leu	Asp	Ala	Arg	Lys	Ile	Arg	Glu	Ile	Leu	Ile	Lys	Ala	Lys		645	650			655	
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50	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp		35	40			45	
	Asp	Asp	Asp	Glu	Asp	Ser	Gly	Ala	Glu	Ala	Ser	Leu	Glu	Val	Leu	Phe		50	55			60	
55	Gln	Gly	Pro	Glu	Asp	Ala	Lys	Asn	Ile	Lys	Lys	Gly	Pro	Ala	Pro	Phe	65	70	75			80	

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	Tyr	Pro	Leu	Glu	Asp	Gly	Thr	Ala	Gly	Glu	Gln	Leu	His	Lys	Ala	Met	
					85					90					95		
5	Lys	Arg	Tyr	Ala	Leu	Val	Pro	Gly	Thr	Ile	Ala	Phe	Thr	Asp	Ala	His	
				100					105					110			
10	Ile	Glu	Val	Asn	Ile	Thr	Tyr	Ala	Glu	Tyr	Phe	Glu	Met	Ser	Val	Arg	
			115					120					125				
15	Leu	Ala	Glu	Ala	Met	Lys	Arg	Tyr	Gly	Leu	Asn	Thr	Asn	His	Arg	Ile	
		130					135					140					
20	Val	Val	Cys	Ser	Glu	Asn	Ser	Leu	Gln	Phe	Phe	Met	Pro	Val	Leu	Gly	
	145					150					155					160	
25	Ala	Leu	Phe	Ile	Gly	Val	Ala	Val	Ala	Pro	Ala	Asn	Asp	Ile	Tyr	Asn	
				165						170					175		
30	Glu	Arg	Glu	Leu	Leu	Asn	Ser	Met	Asn	Ile	Ser	Gln	Pro	Thr	Val	Val	
			180						185					190			
35	Phe	Val	Ser	Lys	Lys	Gly	Leu	Gln	Lys	Ile	Leu	Asn	Val	Gln	Lys	Lys	
			195					200					205				
40	Leu	Pro	Ile	Ile	Gln	Lys	Ile	Ile	Ile	Met	Asp	Ser	Lys	Thr	Asp	Tyr	
		210					215					220					
45	Gln	Gly	Phe	Gln	Ser	Met	Tyr	Thr	Phe	Val	Thr	Ser	His	Leu	Pro	Pro	
	225					230					235					240	
50	Gly	Phe	Asn	Glu	Tyr	Asp	Phe	Val	Pro	Glu	Ser	Phe	Asp	Arg	Asp	Lys	
				245						250					255		
55	Thr	Ile	Ala	Leu	Ile	Met	Asn	Ser	Ser	Gly	Ser	Thr	Gly	Leu	Pro	Lys	
			260						265					270			
60	Gly	Val	Ala	Leu	Pro	His	Arg	Thr	Ala	Cys	Val	Arg	Phe	Ser	His	Ala	
		275						280					285				
65	Arg	Asp	Pro	Ile	Phe	Gly	Asn	Gln	Ile	Ile	Pro	Asp	Thr	Ala	Ile	Leu	
		290					295					300					
70	Ser	Val	Val	Pro	Phe	His	His	Gly	Phe	Gly	Met	Phe	Thr	Thr	Leu	Gly	
	305					310					315					320	
75	Tyr	Leu	Ile	Cys	Gly	Phe	Arg	Val	Val	Leu	Met	Tyr	Arg	Phe	Glu	Glu	
				325						330					335		

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	Glu	Leu	Phe	Leu	Arg	Ser	Leu	Gln	Asp	Tyr	Lys	Ile	Gln	Ser	Ala	Leu	
				340					345					350			
5	Leu	Val	Pro	Thr	Leu	Phe	Ser	Phe	Phe	Ala	Lys	Ser	Thr	Leu	Ile	Asp	
			355					360					365				
10	Lys	Tyr	Asp	Leu	Ser	Asn	Leu	His	Glu	Ile	Ala	Ser	Gly	Gly	Ala	Pro	
		370					375					380					
15	Leu	Ser	Lys	Glu	Val	Gly	Glu	Ala	Val	Ala	Lys	Arg	Phe	His	Leu	Pro	
	385					390					395					400	
20	Gly	Ile	Arg	Gln	Gly	Tyr	Gly	Leu	Thr	Glu	Thr	Thr	Ser	Ala	Ile	Leu	
					405					410					415		
25	Ile	Thr	Pro	Glu	Gly	Asp	Asp	Lys	Pro	Gly	Ala	Val	Gly	Lys	Val	Val	
				420					425					430			
30	Pro	Phe	Phe	Glu	Ala	Lys	Val	Val	Asp	Leu	Asp	Thr	Gly	Lys	Thr	Leu	
			435					440					445				
35	Gly	Val	Asn	Gln	Arg	Gly	Glu	Leu	Cys	Val	Arg	Gly	Pro	Met	Ile	Met	
		450					455					460					
40	Ser	Gly	Tyr	Val	Asn	Asn	Pro	Glu	Ala	Thr	Asn	Ala	Leu	Ile	Asp	Lys	
	465					470					475					480	
45	Asp	Gly	Trp	Leu	His	Ser	Gly	Asp	Ile	Ala	Tyr	Trp	Asp	Glu	Asp	Glu	
					485					490					495		
50	His	Phe	Phe	Ile	Val	Asp	Arg	Leu	Lys	Ser	Leu	Ile	Lys	Tyr	Lys	Gly	
				500					505					510			
55	Tyr	Gln	Val	Ala	Pro	Ala	Glu	Leu	Glu	Ser	Ile	Leu	Leu	Gln	His	Pro	
			515					520					525				
60	Asn	Ile	Phe	Asp	Ala	Gly	Val	Ala	Gly	Leu	Pro	Asp	Asp	Asp	Ala	Gly	
		530					535					540					
65	Glu	Leu	Pro	Ala	Ala	Val	Val	Val	Leu	Glu	His	Gly	Lys	Thr	Met	Thr	
	545					550					555					560	
70	Glu	Lys	Glu	Ile	Val	Asp	Tyr	Val	Ala	Ser	Gln	Val	Thr	Thr	Ala	Lys	
					565					570					575		
75	Lys	Leu	Arg	Gly	Gly	Val	Val	Phe	Val	Asp	Glu	Val	Pro	Lys	Gly	Leu	
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Lys Lys Gly Gly Lys
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Asp Asp Asp Glu Asp Ser Gly Ala Glu Ile Gln Asp Asp Asp Glu Glu
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Gly Phe Asp Asp Glu Glu Glu Phe Asp Asp Asp Asp Asp Glu His
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Asp Asp Asp Asp Leu Glu Asn Glu Glu Asn Glu Leu Glu Glu Leu Glu
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Glu Arg Val Glu Ala Arg Lys Lys Met His His His His His His Ser
100 105 110

Ser Gly Met Ser Pro Ile Leu Gly Tyr Trp Lys Ile Lys Gly Leu Val
115 120 125

Gln Pro Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu
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His Leu Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe
145 150 155 160

Glu Leu Gly Leu Glu Phe Pro Asn Leu Pro Tyr Tyr Ile Asp Gly Asp
165 170 175

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			195					200					205				
	Leu	Glu	Gly	Ala	Val	Leu	Asp	Ile	Arg	Tyr	Gly	Val	Ser	Arg	Ile	Ala	
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	Tyr	Ser	Lys	Asp	Phe	Glu	Thr	Leu	Lys	Val	Asp	Phe	Leu	Ser	Lys	Leu	
	225					230					235					240	
15	Pro	Glu	Met	Leu	Lys	Met	Phe	Glu	Asp	Arg	Leu	Cys	His	Lys	Thr	Tyr	
				245						250					255		
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	Leu	Asp	Val	Val	Leu	Tyr	Met	Asp	Pro	Met	Cys	Leu	Asp	Ala	Phe	Pro	
25			275					280					285				
	Lys	Leu	Val	Cys	Phe	Lys	Lys	Arg	Ile	Glu	Ala	Ile	Pro	Gln	Ile	Asp	
	290						295					300					
30	Lys	Tyr	Leu	Lys	Ser	Ser	Lys	Tyr	Ile	Ala	Trp	Pro	Leu	Gln	Gly	Trp	
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	Gln	Ala	Thr	Phe	Gly	Gly	Gly	Asp	His	Pro	Pro	Lys	Ser	Asp	Leu	Val	
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	Pro	Arg	Gly	Ser	Pro	Glu	Phe	Pro	Gly	Arg	Leu	Glu	Arg	Pro	His	Arg	
				340					345					350			
40	Asp	Gly	Pro	Asn	Thr	Glu	Phe	Ala	Leu	Ser	Leu	Leu	Arg	Lys	Asn	Ile	
			355					360					365				
	Met	Thr	Ile	Thr	Thr	Ser	Lys	Gly	Glu	Phe	Thr	Gly	Leu	Gly	Ile	His	
45			370				375					380					
	Asp	Arg	Val	Cys	Val	Ile	Pro	Thr	His	Ala	Gln	Pro	Gly	Asp	Asp	Val	
50			385			390					395					400	
	Leu	Val	Asn	Gly	Gln	Lys	Ile	Arg	Val	Lys	Asp	Lys	Tyr	Lys	Leu	Val	
				405						410					415		
55	Asp	Pro	Glu	Asn	Ile	Asn	Leu	Glu	Leu	Thr	Val	Leu	Thr	Leu	Asp	Arg	
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15 Claims

1. A method for purifying a protein, comprising steps of:

20 preparing a sample comprising a fusion protein and contaminant proteins, the fusion protein comprising an amino acid sequence of a peptide tag and an amino acid sequence of a target protein, and separating the contaminant proteins and the fusion protein, wherein the peptide tag comprises 12 or more acidic amino acid residues.

25 2. The method for purifying a protein according to claim 1, wherein the peptide tag comprises any of the amino acid sequences of SEQ ID NOs. 1 to 8.

3. The method for purifying a protein according to claim 1 or 2, wherein the peptide tag comprises 18 or more acidic amino acid residues.

30 4. The method for purifying a protein according to any of claims 1 to 3, wherein the isoelectric point of the fusion protein is less than 6.

35 5. The method for purifying a protein according to any of claims 1 to 4, wherein the salt concentration of the sample is 50 mM or more and 500 mM or less.

40 6. The method for purifying a protein according to any of claims 1 to 5, wherein, in the sample preparation step, a vector containing a polynucleotide encoding the fusion protein is introduced into a host cell, and the protein is expressed in the host cell to prepare the sample.

7. The method for purifying a protein according to any of claims 1 to 6, wherein the fusion protein further comprises a cleavable site recognized by a protease, between the amino acid sequence of a peptide tag and the amino acid sequence of the target protein.

45 8. The method for purifying a protein according to any of claims 1 to 7, wherein, in the step of separating the contaminant proteins, the fusion protein and the contaminant proteins are separated using an ion exchange resin.

9. The method for purifying a protein according to claim 8, wherein the ion exchange resin is an anion exchange resin.

50 10. The method for purifying a protein according to any of claims 1 to 9, wherein, the step of separating the contaminant proteins comprises passing the sample through a anion exchange resin to obtain (i) the anion exchange resin to which the fusion protein is bound and (ii) a flow-through fraction comprising the contaminant proteins, and eluting the fusion protein from the anion exchange resin using a buffer with a salt concentration of 600 mM or more.

55 11. The method for purifying a protein according to any of claims 1 to 10, after the step of separating the contaminant proteins, further comprising a step of

separating the fusion protein into the target protein and the peptide tag in a solution, using the protease recognizing the cleavable site.

- 5 **12.** The method for purifying a protein according to claim 11, wherein, in the step of separating the fusion protein, the solution comprising the separated target protein and the separated peptide tag is passed through an anion exchange resin, and

10 (i) the anion exchange resin to which the separated peptide tag is bound, and (ii) a flow-through fraction comprising the target protein are obtained.

- 10 **13.** The method for purifying a protein according to claim 10, wherein the salt concentration of the solution comprising the target protein and the peptide tag is 500 mM or less.

- 15 **14.** A fusion protein comprising an amino acid sequence of a peptide tag and an amino acid sequence of a target protein, the peptide tag comprising 12 or more acidic amino acid residues.

- 15 **15.** The fusion protein according to claim 14, wherein the peptide tag comprises any of the amino acid sequences of SEQ ID NOs. 1 to 8.

- 20 **16.** The fusion protein according to claim 14 or 15, wherein the peptide tag comprises 18 or more acidic amino acid residues.

- 20 **17.** The fusion protein according to any of claims 14 to 16, wherein the isoelectric point of the fusion protein is less than 6.

- 25 **18.** The fusion protein according to any of claims 14 to 17, wherein the fusion protein further comprises a cleavable site recognized by a protease, between the amino acid sequence of the peptide tag and the amino acid sequence of the target protein.

- 30 **19.** A method for producing the fusion protein according to any of claims 14 to 18, comprising steps of introducing a polynucleotide encoding the amino acid sequence of the peptide tag and a polynucleotide encoding the amino acid sequence of the target protein into a host cell, and expressing the fusion protein in the host cell.

- 35 **20.** Use of a peptide tag comprising 12 or more acidic amino acid residues, for the method according to any of claims 1 to 13.

FIG. 1A

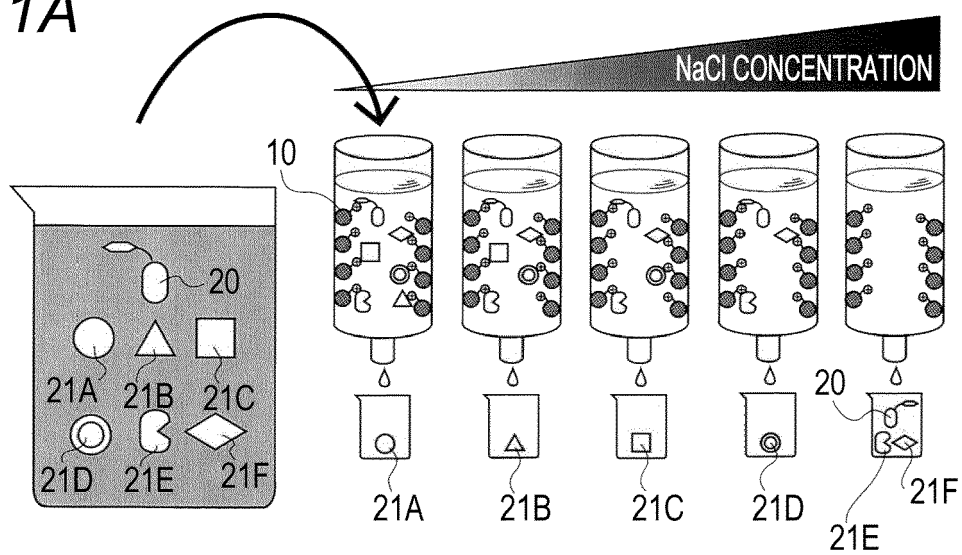


FIG. 1B

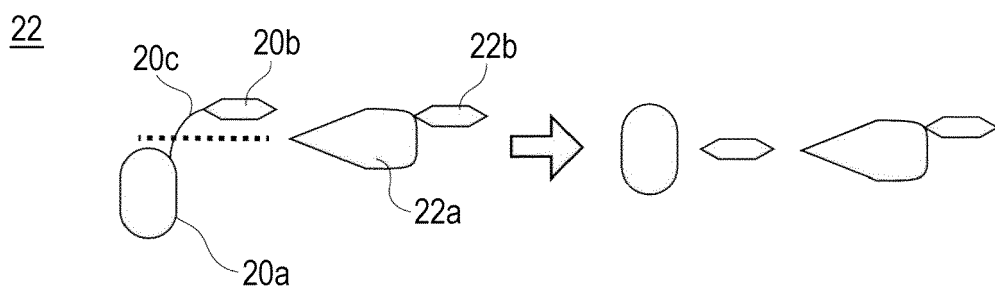


FIG. 1C

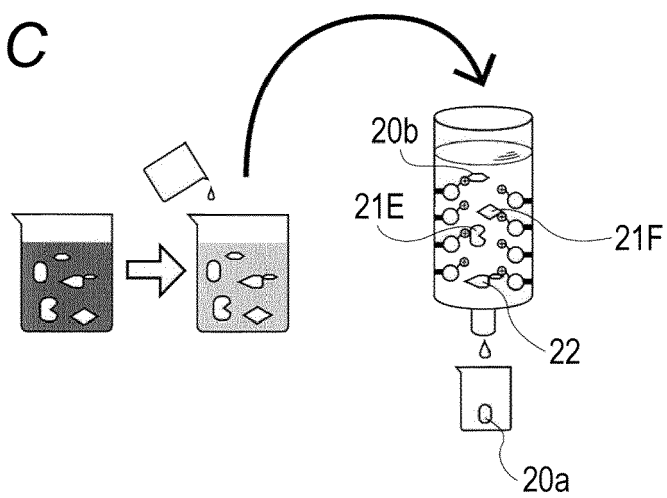


FIG. 2

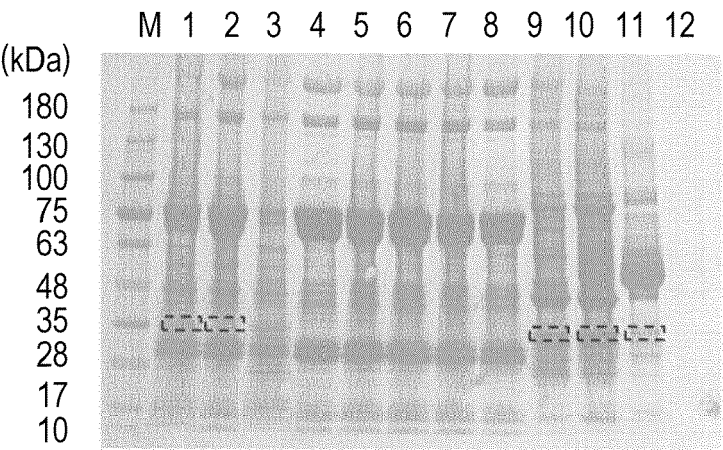


FIG. 3

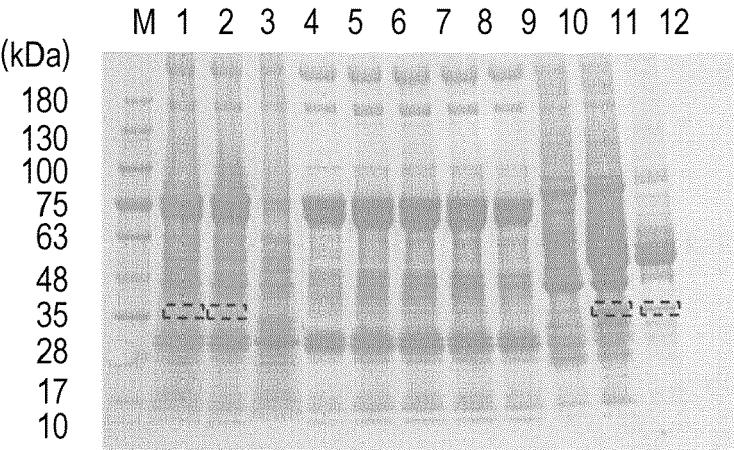


FIG. 4

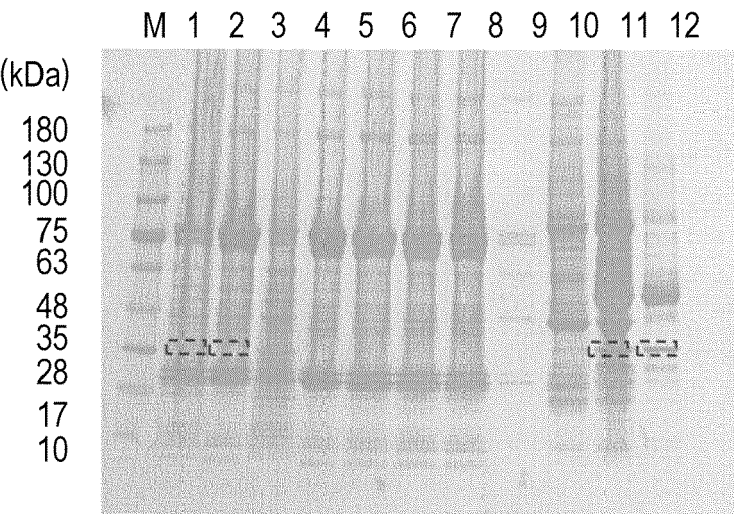


FIG. 5

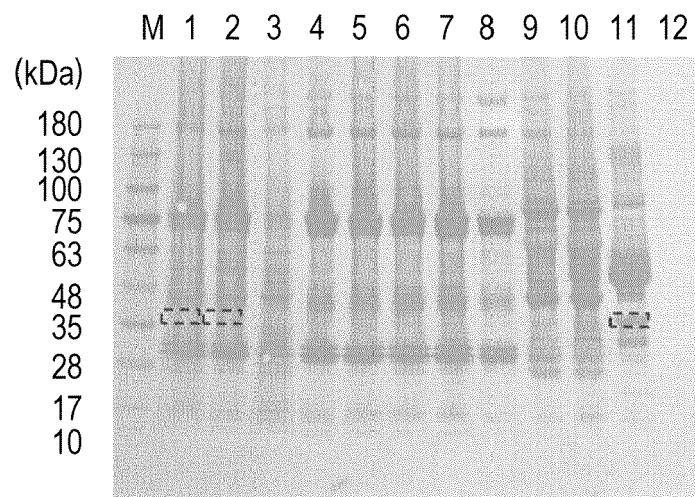


FIG. 6

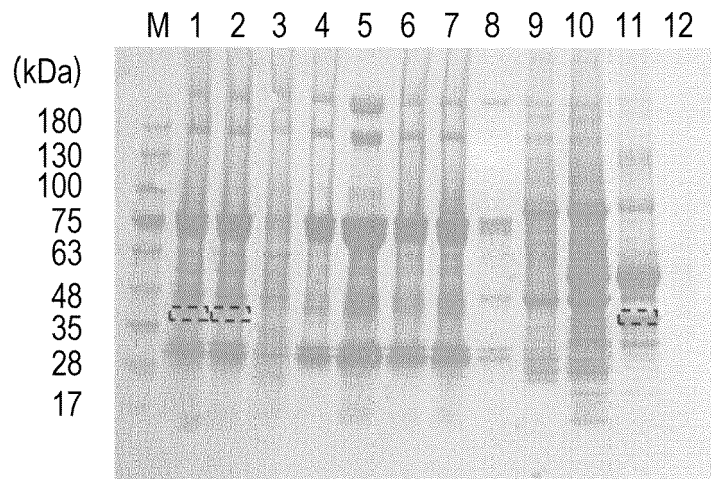


FIG. 7

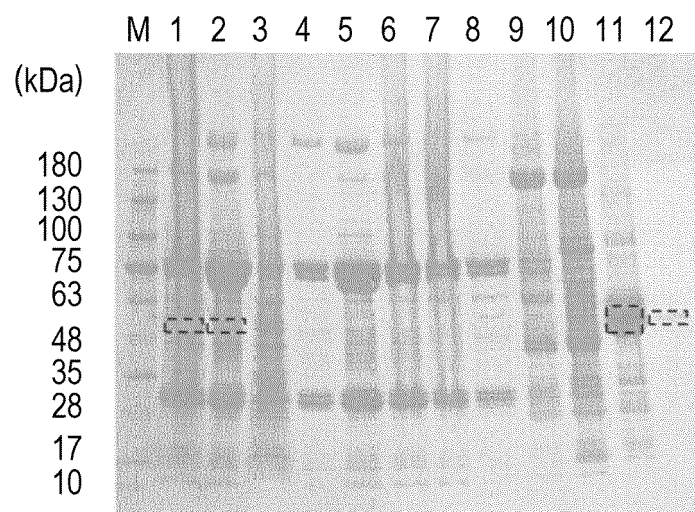


FIG. 8

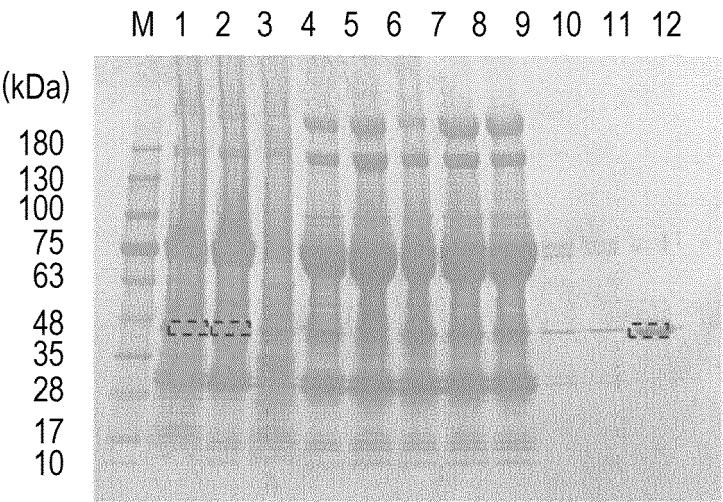


FIG. 9

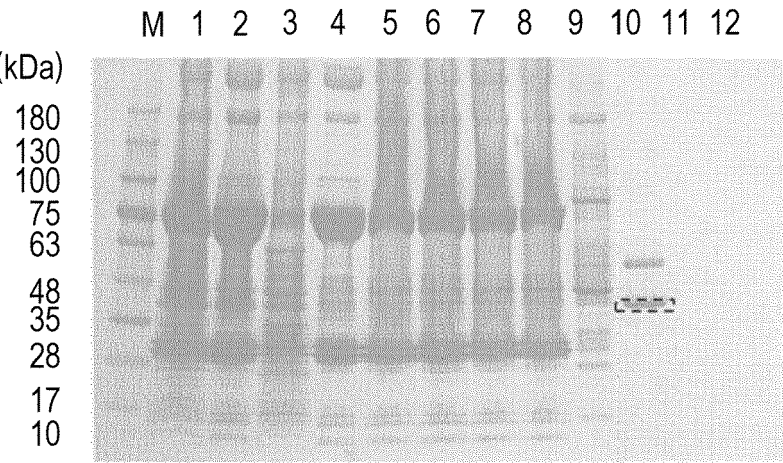
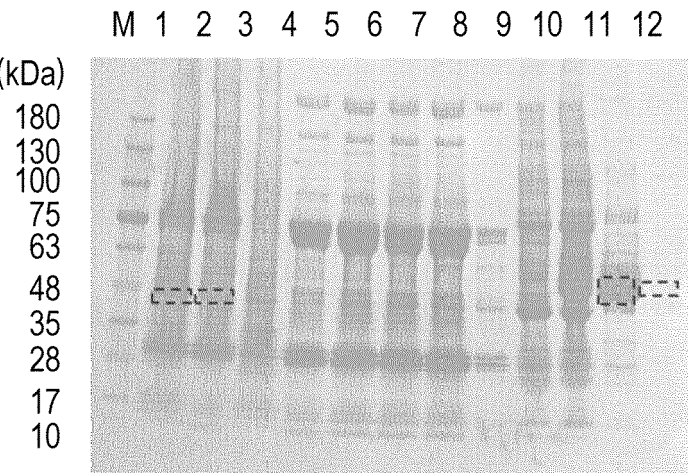
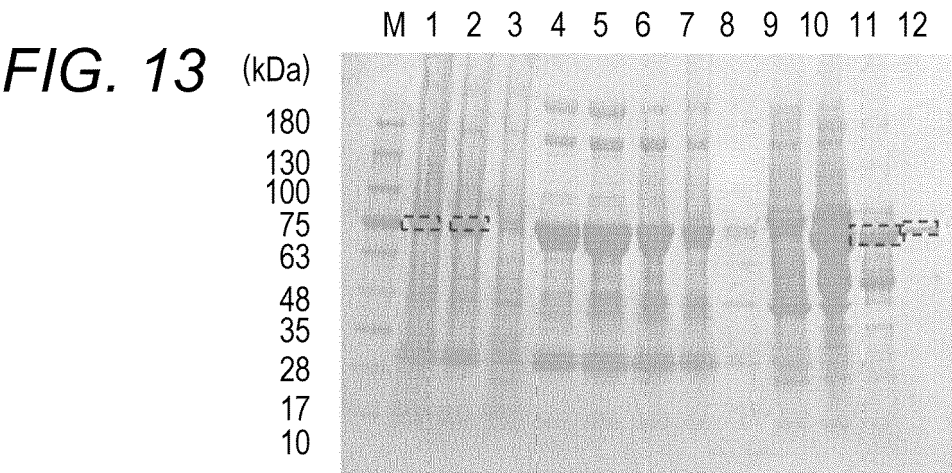
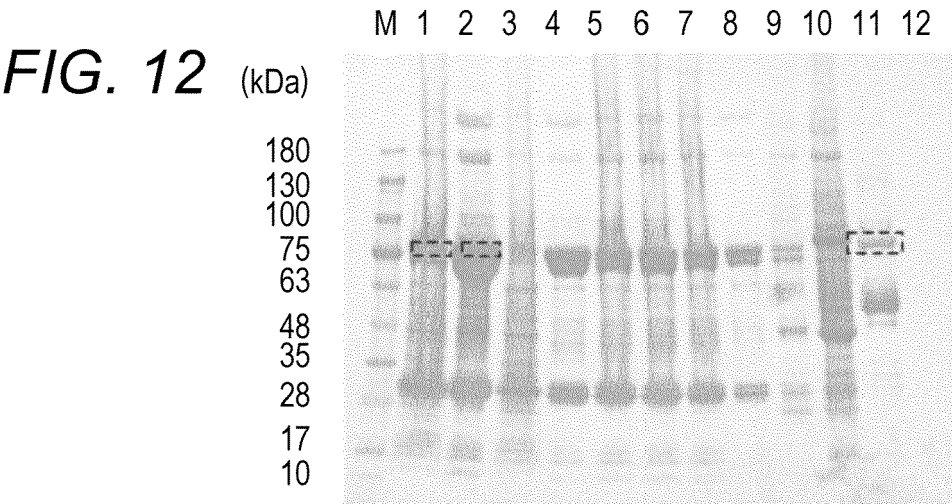
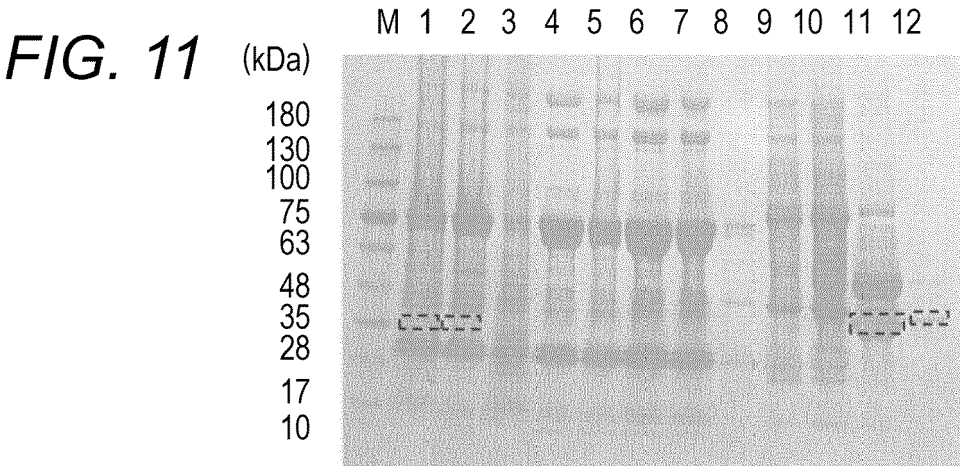
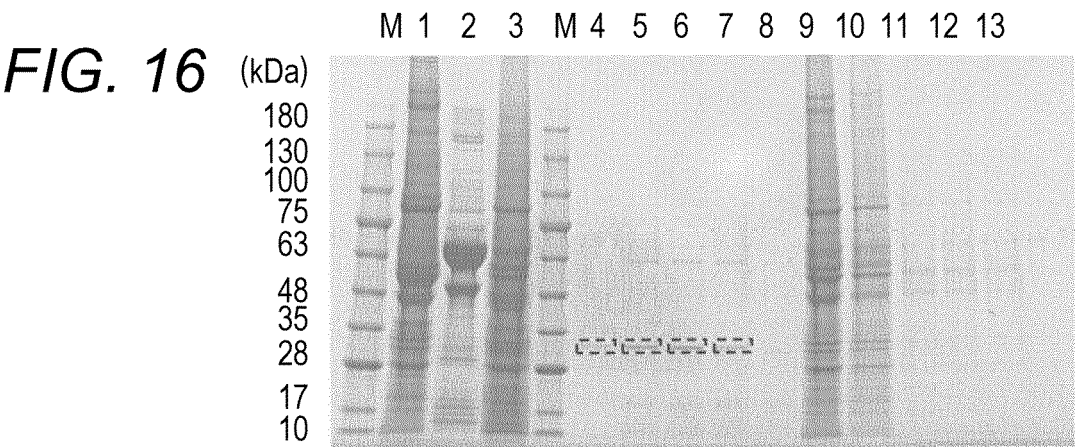
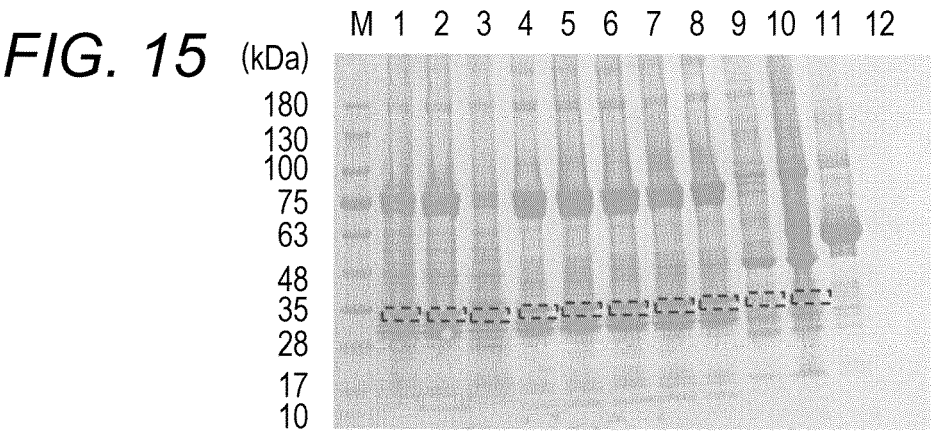
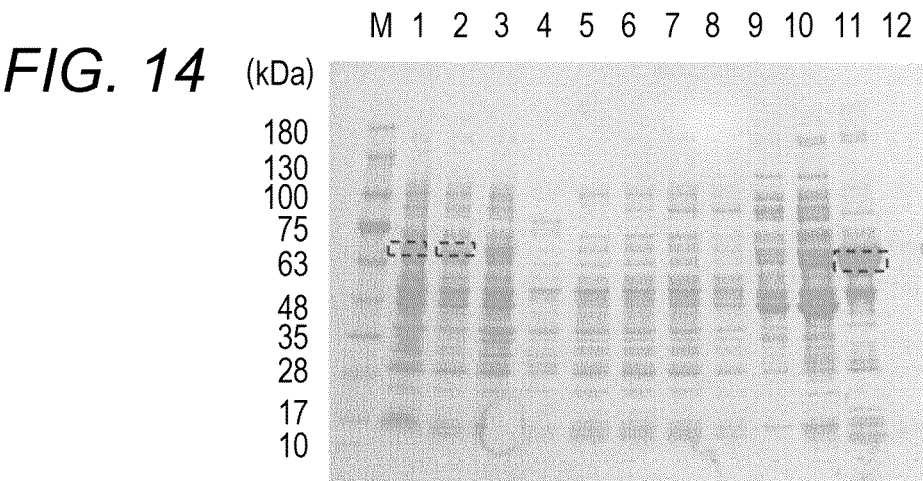


FIG. 10







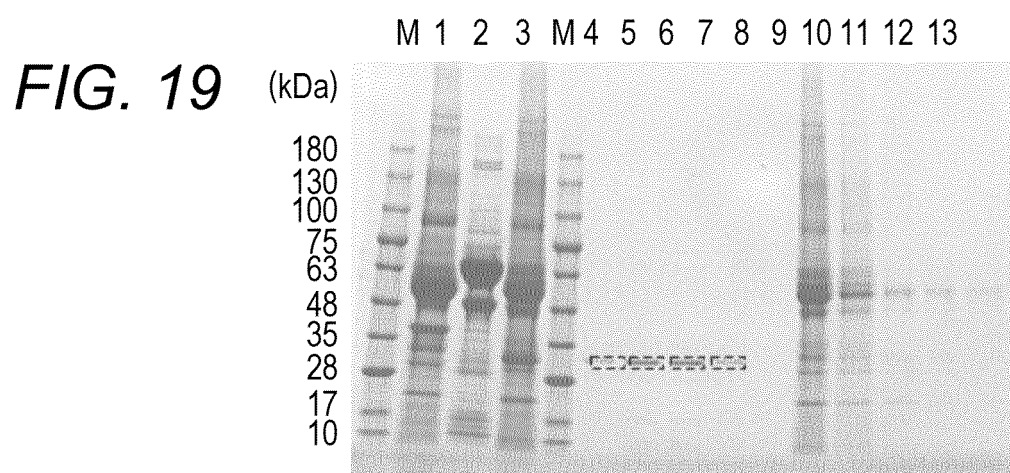
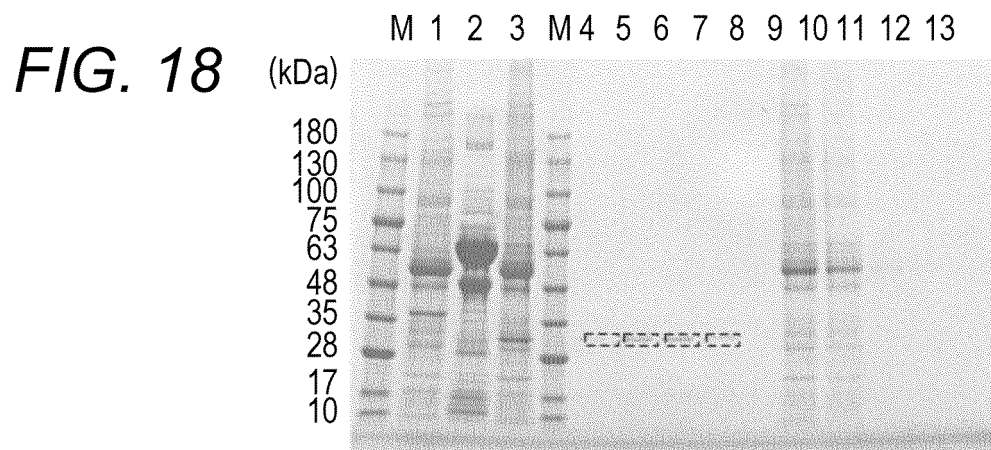
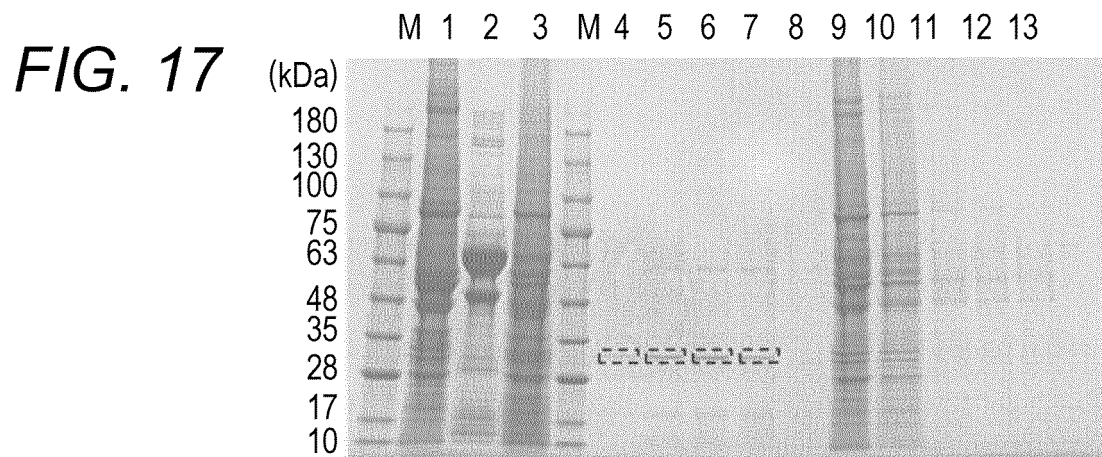


FIG. 20

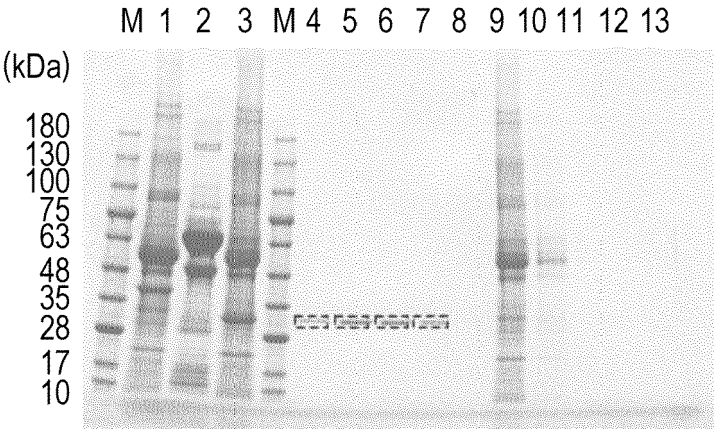


FIG. 21

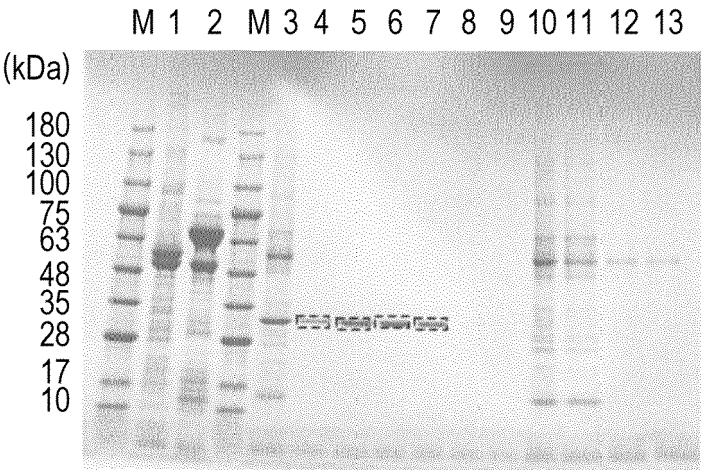
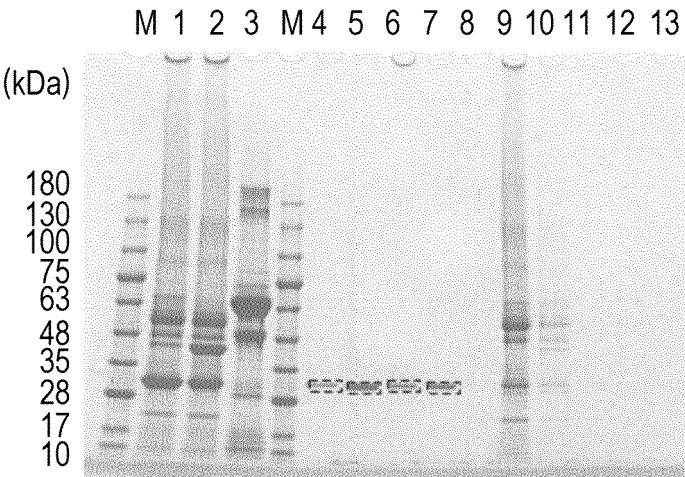
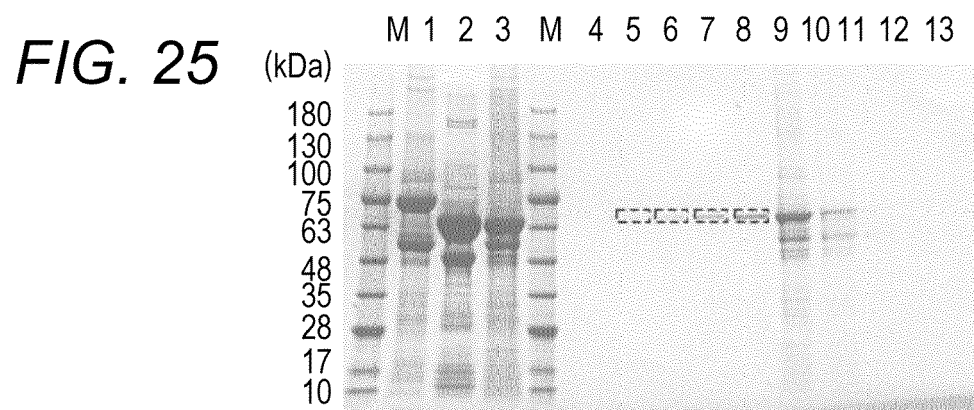
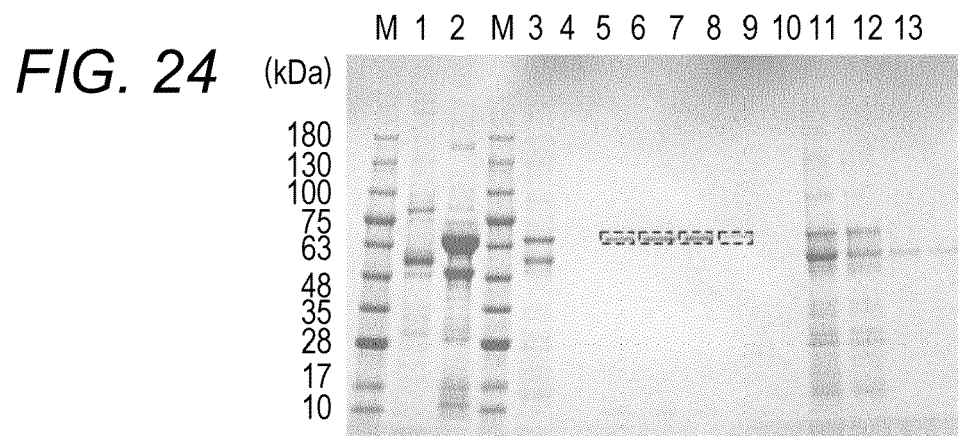
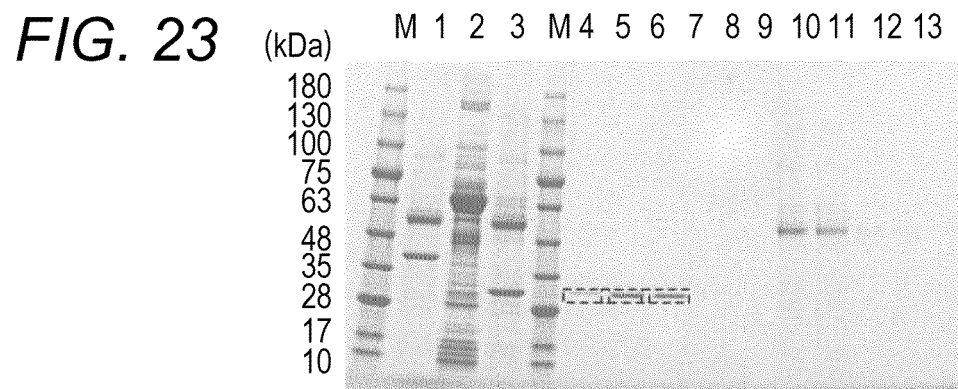


FIG. 22





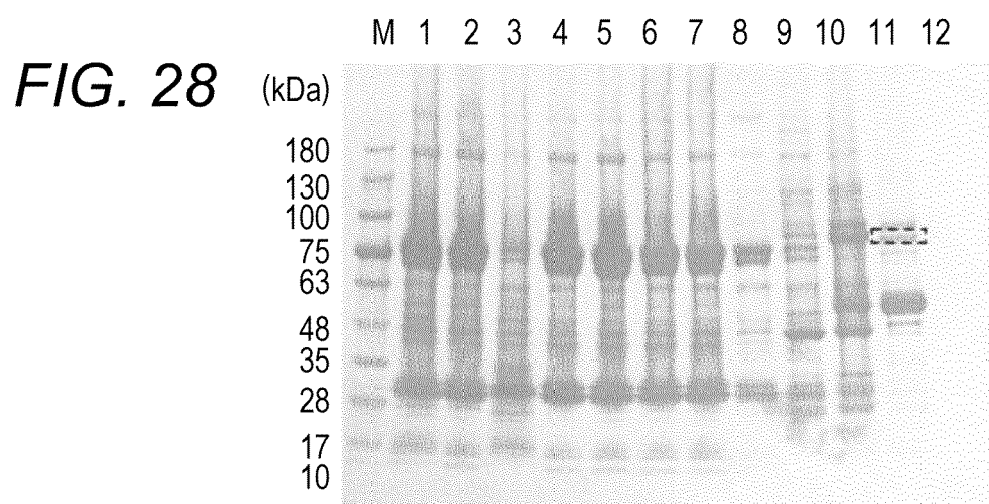
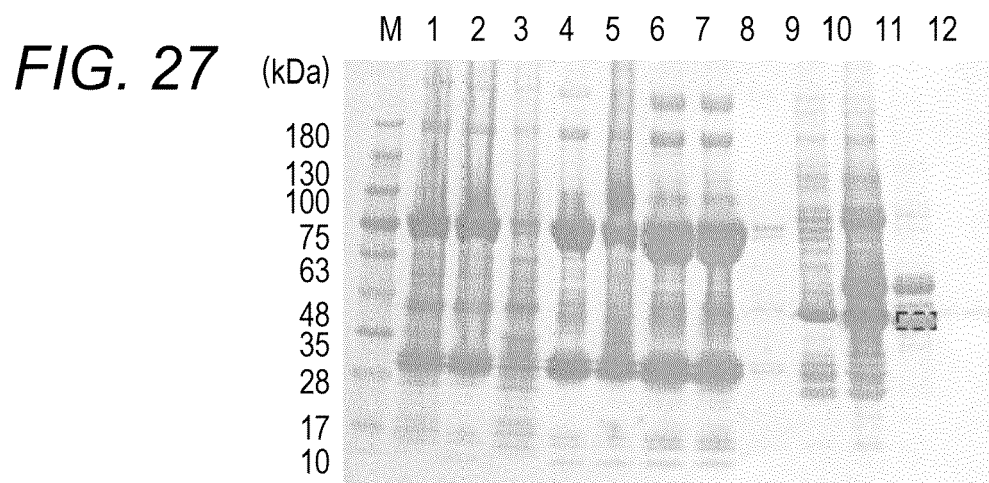
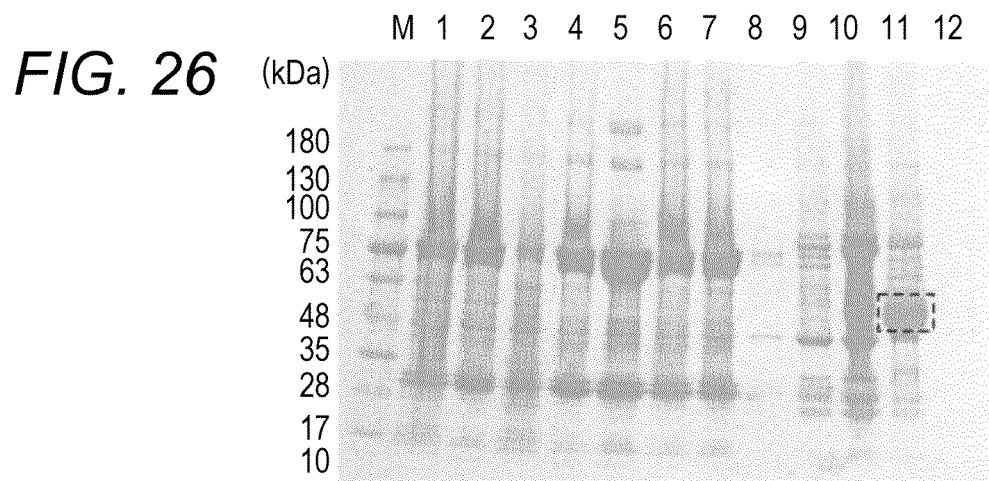


FIG. 29

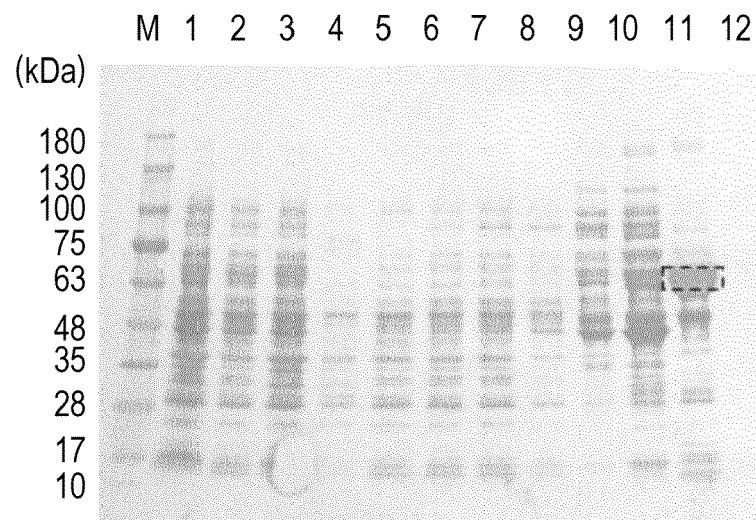
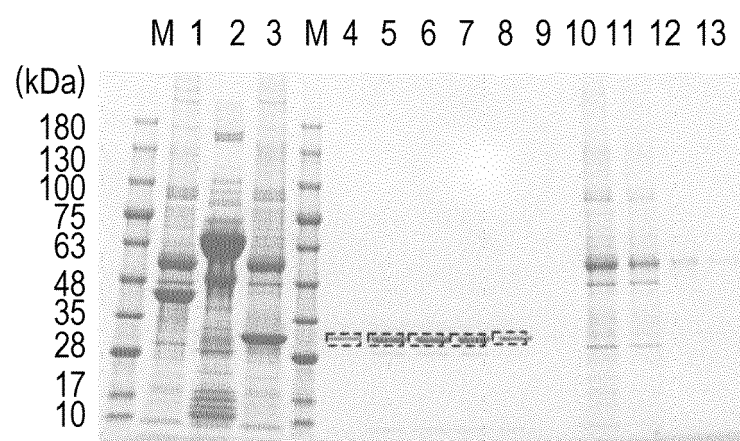


FIG. 30



REFERENCES CITED IN THE DESCRIPTION

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Non-patent literature cited in the description

- **STUBENRAUCH K et al.** Purification of a viral coat protein by an engineered polyionic sequence. *J. Chromatogr. B. Biomed. Sci. Appl.*, 2000, vol. 737, 77-84 **[0002]**